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CD34+ hemopoietic precursor and stem cells traffic in peripheral blood of celiac patients is significantly increased but not directly related to epithelial damage severity

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Key words

CD34+ cell, Celiac Disease, Chronic Inflammatory Disease, CXCR3, CXCR4, Hemopoietic Precursor Cell, iNKT, NFkappaB, Niches, NK cell, Refractory Celiac Disease, Stem cell, T cell, Vbeta8.2

Summary

Celiac disease (CD) is a chronic inflammatory enteropathy of the small bowel resulting from a local TH1-mediated reaction to wheat gliadins and barley, rye and oat prolamins with the development of auto-antibodies to transglutaminases. As well as for other chronic inflammatory diseases, genetic background and environmental factors participate to pathogenesis. An increased traffic of CD34+ hemopoietic precursor and stem cells (HPC) has been reported in peripheral blood (PB) of subjects with allergic diseases that share in their pathogenesis immuno-mediated reactions, genetic and environmental factors. The aim of the present work was to investigate the CD34+ cell traffic and H2 / H1 polarization of lymphoid T-cell lineage, in the peripheral blood of subjects with CD, by means of flow-cytometric techniques. Group A of control was of 20 healthy subjects, aged 5 to 58 years. Study population (Group B) was of twenty-eight patients, all females aged 13 to 70, receiving firstly a CD diagnosis at the SS Annunziata Hospital Digestive Physiopathology Out-standings' by means of clinical, serologic and small intestinal biopsy findings. Peripheral CD34+ HPCs were significantly increased in Group B (median value 0.16) when compared with Group A (median value 0.03) (p 0.0001) but did not correlate either with anti-transglutaminase (tTG) antibody levels (IgA: p 0.226; IgG: p 0.810) or with histological damage severity (p 0.41) that, on the contrary, was significantly related with anti-tTG IgA antibodies (p 0.027). Celiac circulating CD3+CD4+ lymphocytes expressed a chemokine-receptor pattern Th2-skewed in all but three patients investigated. Concluding, the CD34+ HPC highly increased peripheral traffic observed in celiac disease appears more related to a basic and emerging as common defect shared by chronic inflammatory diseases than to the gliadin-specific Th1 local reactions. Data are consistent with a potential NFkappaB deficiency and consequent prevalence of apoptotic versus survival programs leading to excessive cell-death; to replace lost cells a supplementary bone-marrow derived precursors supply, further to that physiologically provided by the gut stem cell "niches" that are cryptopatches, could be required.

Introduction

Immuno-Mediated Inflammatory Diseases (IMID) such as allergic and auto-immune diseases have been interpreted, for more than thirty-five years, as the result of a TH2 cell reaction for allergy and TH1 for auto-immunity respectively, according to a divergent view of TH2/TH1 paradigm, where cell polarization was thought to depend on the nature of antigen/allergen. Doubts concerning such interpretation of the paradigm have thus generated recurrent debate during latest years (1-4).
Later a linear version of the paradigm has been proposed, not only for T-cell lineage but also for NK and dendritic cell (5-11), where H2, H0 and H1 were, simply, the early, intermediate and late stages of an effective cell-lineage differentiation/maturational events (cell-lineage physiological ontogeny). Under this optic the very low amount of TH1 cells characteristic of allergic subjects has been interpreted as the consequence of a disturbed ontogeny due to the difficulty in the progression of differentiation/maturation at the *early/intermediate/late* stages (12-15), sustained by genetic defects impairing the maturational citokine IL12 and IFNs receptors (16-20).
Most recently, T cell-mediated tissue damage has been referred to a novel identified T cell sub-population, termed TH17, generated during an altered T-cell lineage differentiation defective in IL2 and/or IL12 expression (pathway TH17) (21-23) leading to the first major revision of the above mentioned TH2/TH1 hypothesis (24).
TH1 cells, whose role has long been considered paramount in the pathogenesis of autoimmune diseases, are now credited to antagonize Th17 pathway (24).
The large body of evidence regarding monogenic defects underlying a new and wide identified chapter of primary immunodeficiencies, has been systematically reviewed, with special attention to cell and antibody defected maturational processes linked to a disturbed homeostasis of both the innate and adaptive immune systems (20).
Hematopoietic Precursor (HPC) and diverse white-lineages-committed Stem Cell (SC) have been, furthermore, recognised as involved in systemic and local aspects of allergic inflammation (25-41) and their traffic in Peripheral blood (PB) has been shown to be significantly increased in allergic subjects but not during infectious inflammation (42).
The aim of the present study is to investigate the CD34+ HPC and stem cells traffic in PB and the h2/h1 associate pattern of chemokine receptor CXCR4 (H2) and CXCR3 (H1) expression by peripheral T-lymphocytes in subjects suffering from Celiac Disease.

Methods

Subjects

HPC-CD34+ and chemokine-receptor expression have been investigated in PB samples of two groups of subjects, randomly recruited and processed in a blinded manner.
Group A (control) was of twenty healthy subjects (10 males and 10 females aged 5 to 58 years, mean age 24. 5 years) negative for clinical history of any chronic inflammatory diseases.
Group B (pathologic) was of twenty-eight patients (all females aged 13 to 70 years, mean age 32.5 years) receiving the first diagnosis of CD at the *SS Annunziata* Hospital Digestive Pysiopathology Out-standings' by mean of the serological evaluation of anti-gliadin IgG and IgA (AGA-IgG and IgA), anti-trans-glutaminase IgG and IgA (tTG-IgG and IgA) antibodies and of small intestinal biopsy, according with Corazza-Villanacci severity classification (A: increased number of Intra-Epithelial Lymphocytes but no structural modification of the mucosa architecture, B1 = moderate mucosa damage, B2 = severe mucosa damage with not identifiable villous) (43).
Oral informed consent or assent (from under 18 years old) was obtained from the subjects and parents/guardians at the time of recruitment.

Serological assays

In vitro quantitative measurement of serum IgA and IgG antibodies specific for gliadin has been performed with *ImmunoCap Gliadin IgA/IgG* commercial kit purchased by Phadia AB (Uppsala, Sweden), according to manufacturer recommendations. Results were expressed in mg/l. Data have not been reported since irrelevant.
In vitro quantitative measurement of serum IgA and IgG antibodies specific for human-recombinant tissue transglutaminase (tTG) has been performed with *Celikey* commercial kit purchased by Phadia AB (Uppsala, Sweden), according to manufacturer recommendations. Results were expressed by a quantitative elaboration of examined sample versus a calibration curve. The expression Units/ml were the ratio between $OD_{sample}/OD_{cut\text{-}off}$.
Whit a cut-off value of 6 U/ml clinical sensibility is 96% and clinical specificity is 99%.
Values have been considered as positive from values of 8 U/ml onwards.

Flow cytometry

To investigate the entire pool of immature mononuclear circulating cells, independently from lineage commitment (mono-potent stem cell) or un-commitment (multi-potent hematopoietic precursor cells), a simple method based on a partial modification of the Milan Protocol of Peripheral Blood CD34+ Cell Estimation was followed, as previously described (34, 42).

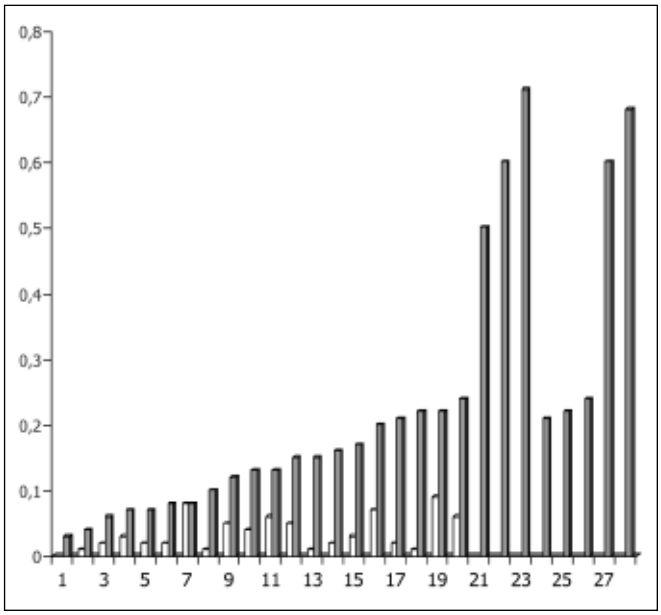
Peripheral or cord blood venous samples were collected in ethylendiaminetetraacetic acid (EDTA) tubes. Three aliquots of 50 ml of whole blood were incubated, at 4°C for 25 min in the dark, one with 15 μl of phycoerythrin (PE)-conjugated murine monoclonal antibody (MAB) specific to CD34 molecule, one with PE-conjugated MABs specific to unrelated molecules to human leukocyte antigens, and one as unlabeled control. Monoclonal antibodies were from Becton Dickinson (BD), Milan, Italy. PE anti-CD34 MAB was the Anti-HPCA2, Clone 8G12 (BD), suitable for routine use. Then, 2 ml of red blood cell lysis buffer (BD, Italy) were added to each tube and incubate at room temperature for 10 min, in the dark. After lysis cells were washed twice with 2 ml of cold phosphate buffered salt solution (PBS) (BD, Italy) at 1,200 revolution per minute (rpm) (300 g) for 7 min at 4°C and then resuspended in 500 μl of PBS.

Cells were analysed using a FACSCalibur flow cytometer equipped with Cell Quest software. Three data parameters were acquired and stored in list mode files: linear forward scatter (FSC) (vertical axis), linear side-angle scatter (SSC) (horizontal axis) and log PE fluorescence, by gating whole viable cells (Fig.). Since our aim was to enumerate both $CD34^{bright}$ and $CD34^{dim}$ cells, the setting of the fluorescence analysis region 2 (R2) has been fixed with the lower limit at 10^2 avoiding any compensation. For each measurement 10,000 events were acquired. Results have been expressed as percentage of positive cells.

CXCR4 and CXCR3 expression on CD2+CD4+ and CD3+CD4+ cells has been performed using mouse anti-human Monoclonal Antibodies, as follow: CD2 FITC, CD3 PerCP (*Peridinin-Chlorophyll-Protein*), CD4 FITC or PerCP , all purchased by BD.

CD184 PE, clone 12G5, was a mouse IgG2a specific for CXCR4 and CD183 PE, clone 1C6/CXR3, was a mouse IgG1 specific for CXCR3; both monoclonals were supplied by Pharmingen. Cells were processed and analysed by current unmodified three-colour-immuno-fluorescence techniques; results expressed as percentage of cells positive.

Figure 1 - Peripheral blood CD34+ precursor and stem cells are significantly (p 0.0001) increased in celiac disease population (grey bars, 28 subjects, median value 0.16) when compared to control group (white bars, 20 healthy subjects, median values 0.03)



Chemokine receptor (CKR) association with TH-cell polarization

Although none of CKRs can univocally identify TH2 or TH1 polarization, because of the diverse nature of naïve, memory and effector sub-populations, as well as the flexibility of some expression programs, CXCR4/CD184 has been proved to be up-regulated by IL4 (44, 45) and down-regulated by IFNγ (45) and has been described as preferentially associated with TH2 cells (44-46) also in T-cell lymphomas with Th2+ immunophenotype (47).

CXCR3/183 expression, on the contrary, appears to mark the IFN-gamma dependent terminal differentiation processes from naïve to memory cells also in B-lineages (48) and thus has been preferentially associated with a TH1 profile (49, 50), particularly with TH1-activated cells transmigrating into tissue sites of inflammation in autoimmune diseases (51-54).

Statistics

Given the not-normal distribution of continuous variables, data have been processed using non-parametric

tests. Comparison between CD34+ peripheral cells circulating in normal versus pathologic groups has been performed by the *Wilcoxon* signed-rank sum test for independent samples. Relationships potential between peripheral CD34+ cell increased traffic and serum auto-antibodies specific to Gliadin, Transglutaminase have been explored using *Spearman*'s rank correlation and with histological damage of the intestinal mucosa using χ^2– test. A p value < 0.05 was considered statistically significant.

Results

CD34+ cell values in PB of healthy subjects (Group A) ranged from 0.01% to 0.09% with a median of 0.03. CD34+ cell values in PB of subjects with Celiac Disease (Group B) ranged from 0.03% to 0.70% with a median of 0.16 and thus resulted significantly increased ($p<0.0001$) when compared with (Group A) (Fig. 1).
Comparisons between Group B CD34+ cells and serum anti-tTG IgA and IgG values were not significant since rank-correlation resulted 0.233 ($p = 0.226$) and 0.051 ($p = 0.810$), respectively.
Equally, χ^2 – test between CD34% categorised with two values major of the median (16) and minor versus histological damage severity [A vs (B1 and B2)] resulted not significant with a $p = 0.41$.
Spearman's rank correlation between serum IgG-anti-tTG and tissue damage (histology severity score) was negative ($p = 0.534$) while IgA-anti-tTG correlated significantly ($p = 0.027$) with grade B of mucosal damage.

The expression of peripheral pattern of chemokine receptors, among CD4+ lymphoid populations (T-cell + a subset of Natural Killer) marked by CD2, was always skewed to h2 (CXCR4) with the exception of one subject . Among CD3+CD4+ T-cell population only three subjects showed a percentage of cells expressing the h1/associated receptor CXCR3 major than the h2/associated (CXCR4) (Fig. 2).

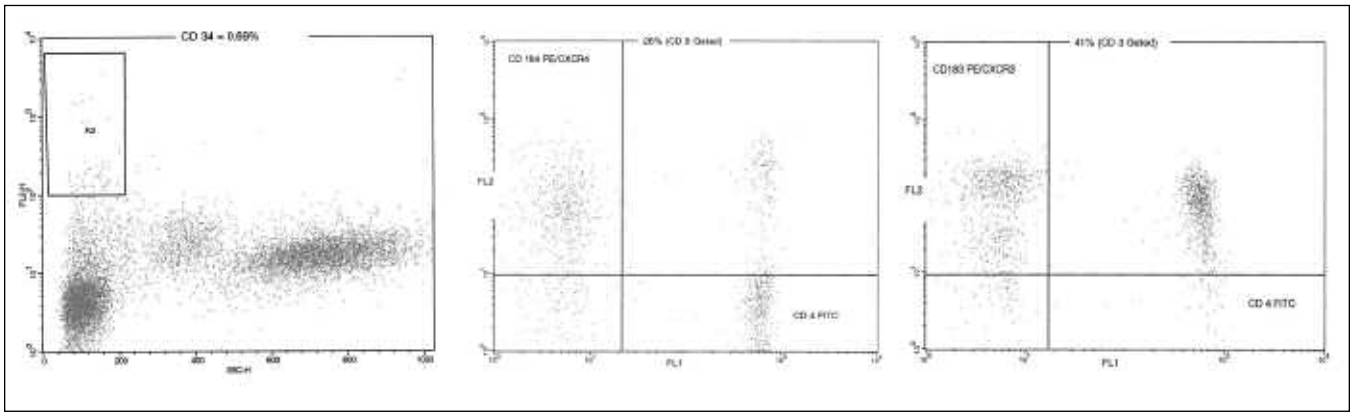
Discussion

Results from the present work have to be discussed in a complicate and fast moving background involving physio-anatomical, immunological and epithelial homeostatic mechanisms.

Stem cell niches in mammals

Accumulating evidence indicates that almost every adult tissue contains stem cells clarifying how cells and microenvironment operate to maintain homeostasis in self-renewal tissues.
Bone-marrow, once regarded as the major if the unique source of HPC and stem cell in the body, has been recently proposed to be a reservoirs of stem cell populations beside the local stem cell niches (55-58).
Gastrointestinal stem cell niches provide, under normal condition, to the physiological replacement of epithelial cell lineages undergoing to a high-rated turnover. Stem cells reside, mainly, in crypts that represent the proliferat-

Figure 2 - Even if celiac patients had a peripheral lymphocyte CD3+CD4+ pattern of KCRs expression prevalently Th2-associated, CD34+HPCs moved significantly also when peripheral T-cells were Th1-polarised, as in the case showed. From left: % of PB CD34+ mononuclear cells, CD3+CD4+ CD184+ (CXCR4 H2-associated), CD3+CD4+ 183+ (CXCR3 H1-associated)



ing compartment when villous the differentiation one, receiving cells from crypts (59, 60). A bone marrow contribution to regeneration of intestinal myofibroblasts has been described during epithelial reparative processes (60-62).

The gut as extra-thymic site of lymphocyte differentiation

After the first report of T-cell maturation in the human gut by Marie Louise Hammarstrom et al. in 1995 (63) and the consequent initial controversies, a large and compelling body of evidence has been cumulated to regard gut as the Thymus substitute-complementary organ for T-lymphocyte precursors development and selection (64-69). In absence of thymopoiesis, in fact, T-cells are nevertheless present principally in gut. The elegant model of athymic/euthymic mice furthermore indicates that in euthymic animals the alternative lymphopoietic pathways are repressed except in conditions of severe lymphocyte depletion (69, 70).
The question about the origin of the lymphoid precursors subsequently arose. In human gastrointestinal tract, hemopoietic stem cells (HSC) CD34+ CD45+ were detected both in the lamina propria and in the epithelium, localized in crypts and in villous.
Most of the HPC were CD34+, CD45+ but c-Kit negative, indicating ongoing differentiation and expressed the CD7 marker of lymphoid lineages. Almost 50% HSC were positive for CD56 in the epithelial layer when only few in lamina propria, suggesting that gut epithelium could be a preferential site of lymphoid Natural Killer (NK) differentiation (71).
Recently the issue has been extensively reviewed (72, 73), with a special attention to the subset of IntraEpithelial Lymphocytes (IELs) that constitute a unique population expressing the T-cell receptor (TcR) alpha beta or gamma delta but CD8 alpha alpha homodimers. Such a T-cell subset is believed to be regulatory, to appear downstream the transitional double positive population CD4+CD8+ (DP) and that could originate by immature but lymphocyte-lineage committed thymic emigrant cells in euthymic mice or by HSC harboured in cryptopatches in athymic animal (74).
Gut criptopatches thus appear to constitute a major component of a physiological system capable of sustaining the entire ontogeny of T-cell lineages phylogenetically less evolved with respect to lineages educated in the thymus.
Thymus-derived cells may have selected to process foreign antigens bearing a wide diversity of TcR diversity and are particularly active in youth when extrathymic T-cell lineages appear to recognize self-reactive abnormal cells and act mainly as immunoregulatory populations in site exposed to antigenic overload. Extrathymic T-cells are few in youth but increase with aging in parallel with thymic involution. Under condition of lymphocytes deficiency as stress and pregnancy this compartment appears expanded even in youth. Taken together mentioned features suggest that T-cells associated with the small intestine epithelium could represent the ultimate evolutionary step of innate immunity bordering and supporting adaptive immunity (75, 76).

Cell-mediated immunological tolerance at glance

The identification of different subsets of regulatory lymphocyte as well as of different cellular signalling molecules and related pathways could be considered one of the most important progress in a field crowded of actors.
Establishment of central tolerance critically depends on AIRE (autoimmune regulator gene) transcriptional activity that allows the thymic medullary epithelial cells (TEC) expression of tissue-restricted self-antigens and the consequent negative selection of the T-repertoire thorough clonal deletions of auto-reactive T-cells, before thymic egression. The developmental expression of AIRE final products on mature medullary TEC appears linked to the cell effective differentiation/maturation dependents on non-classic members of the IkappaB inhibitors family that contrast apoptotic signals at the intermediate stage of the development. Perturbing such a signals pathway has been reported to be associated with a breakdown of central immune tolerance (77-80).
Peripheral tolerance is more complex because of an excessive suppressor activity leads to immunodeficiency or cancer whilst a loss of the function generate autoimmunity; maintaining homeostasis thus requires a perfect balance between action and resting.
Several types of T-regulatory cells (Treg) have been described in last decades generating difficulties in the comprehension of their activity. Among the many, the best characterized are those residing in CD4+ subset that are CD4+CD25+ Treg and type 1 regulatory (Tr1) cells.
Tregs are natural occurring cells generated mainly in the thymus, displaying a highly diverse TcR repertoire (81) and acting in a singular fashion because their activation is antigen-specific but inhibition of effector T-cells is antigen-not-specific (bystander) and cytokine-independent but mediated thorough cell-cell contact (81-84) .
Tregs have been also studied in cord blood (85, 86) rising

the question about a major potential role of such cells in the tolerance induced by CD34+ stem cells (87, 88).
The major feature in CD4+CD25+ Treg subset is the expression of the forkhead transcription factor Foxp3 (Forkhead box P3); interestingly such box has been identified in adenocarcinoma cells as capable of inducing anergy of co-culture T-cell as possible mechanism of cancer immune evasion (89).
Tregs suppress a variety of response including CD4 and CD8 cell proliferation, IFNgamma production, CTL activity (83-85) and appear to regulate NK activity, hampering the generation of mature forms that together with immature dendritic cells guarantee at the periphery a quiescent status of the immune cells (90).
When receptors belonging to the Toll Like family (TLRs) are engaged by pathogens, Tregs are down-modulated allowing an effective immune reaction (91, 92).
Type 1 regulatory cells (Tr1). Opposite to natural occurring Tregs up-regulated by H2 cytokines IL4 and IL13 (82) or by TGF-beta + IL2 (83) and operating suppression in a bystander mode, Tr1 appears a more differentiated cellular subset induced by antigen stimulation and IL10 secreted by immature dendritic cells (DC2); Tr1 regulatory activity is Ag-specific and mediated by IL10 production and thus such cells have been defined *adaptive* to render the concept of specific-suppression even if T-cell Receptor, once expressed, does not undergo further modification as B-cell-receptors do (93, 94). Tr1 population thus appears to be the major candidate as peripherally induced antigen/allergen specific suppressor cells generated, in a tolerogenic environment, with effective low-dose regimens of specific immunotherapy.
A third regulatory population, apparently distinct, but closely related with Tregs and Tr1, is the Th3 subset. Th3 cells are characterized by the production of TGF-beta, and exert their suppressor activity via TGF-beta secretion as well as Tr1 via IL10 secretion (95-97).
Whether Tregs, Tr1 and Th3 cells are three distinct T-subsets with distinct genetic programs or, on the other hand, they represent downstream products of a plastic T-cell lineage proceeding into sequential developmental stages allowed by apoptosis repression induced by different extrinsic co-stimulators (local microenvironment cytokines), it is matter of future evidence when more data from human will be available.
Singularly, human CD25+ regulatory cells express alpha4beta-integrin as homing receptors (98) as well as cord blood CD34+ HPCs (99) and short developmental program cell lineage such as mast cells (100).

Natural Killer T subsets and their regulatory activity: NK-regs

The positive answering to the recently posed question: do "NK-reg cells" exist ?" (100) is from evidence cumulated during the last years.
In 1996, Researchers from The Jefferson M.C. of Philadelphia, described a functionally immature human NK subset, not cytotoxic, expressing only the NKR-P1A receptor but negative for all the other differentiation antigens of mature cells (NKR-P1A+ CD56- CD16-); this subset developed the effector functions when stimulated with IL12, concomitantly with the expression of CD56 (102). Exactly ten years later data have been confirmed by Stanford Researchers (103).
A more accurate knowledge of NK cells immunobiology has recently reappointed the attention over a paramount potential of such a Lymphoid subsets in maintaining immune homeostasis.
Ontogenetic studies suggest that NK progeny rising from CD34+/Lin- precursors, in absence of cytokine/cell-contact specific stimuli, expresses Killer Immunoglobulinlike Receptors (KIR) and is negative for the lectin-receptor CD94 (104), evidencing a non-random sequence of receptor acquisition. IL12 stimulation (105), in fact, induces the expression of the CD94/NKG2-A that is an inhibitory receptor with respect to the NKG2-D, among the products of several gene-subfamilies of lectin-like receptors associated with NK gene complex located on chromosome 12.
The CD94/NKG2 family of receptors is composed of members with activating (NKG2-D) or inhibitory (NKG2-A) capabilities and initial information from clinical condition of excess of tolerance or stimulation is growing in literature (106). NK cells from chronic HCV subjects have been reported to be skewed toward a CD94/NKG2A expression with respect to normal controls and incapable of inducing differentiating changes on immature dendritic cells (iDC) necessary to sustain an effective HCV-clearance (107).
On the other hand, while a direct role for NKG2D-activating receptor has been proposed for CD villous atropy (108, 109), a deficiency of the gut NK population bearing the inhibitory receptor has been associated with experimental autoimmune diabetes (110).
Of major interest is, furthermore, the highly significant ($p < 0.001$) deficiency of a particular *invariant NKT-subsets*, recently reported in Crohn's disease and ulcerative colitis (111), and in celiac disease (112); whilst the role potential of iNKT cells has been matter of controversies with

respect to asthma and allergy (113), evidence suggests a constant reduction in bowel inflammatory disease.
Invariant NK T-cells have been reported, paired to the alpha 14/24 chain, to use the Vbeta 8.2 chain (114); singularly, the Vbeta 8.2 + subset resulted the unique significantly reduced among the Vbeta-repertoire of T circulating cells of a symptomatic mite-allergic population (115), raising the question as to whether such a defect may represent a common feature of immune-mediated inflammatory condition.
Since it is known that germline transcription of the unrearranged Vbeta 8.2 gene is an early event of lymphocyte development, at the stage of precursors, long before Vbeta families rearrangement (116), it has been recently proposed that early lymphoid cells could use a pre-TcR structure based on Vbeta 8.2 gene espression (117).
The ontogeny and function of iNKT are strictly dependent on NF-KappaB signalling (118). Maturation of iNKT precursors depends on the NF-kappa B induced activation of survival program (e.i. up-regulation of Bcl-2) and repression of apoptotic program (e. i. down-regulation of Fas) (119, 120).

Toll-Like Receptors and NF-kappaB/IkappaB regulatory system

NF-kappaB controls a network of genes including those for immune response, cell adhesion, differentiation, proliferation, apoptosis and angiogenesis.
Phylogenetically ancestral, NF-kappaB has been perfectly conserved through evolution as an evidence for a master role in the regulation of the many biological activities above mentioned.
Resting in normal condition because constantly controlled by its inhibitor I-Kappa-B, an activation of the transcription factor NF-kappaB is allowed by the ubiquitin-dependent degradation of the inhibitor I-kappa-B, induced by stimulus-dependent phosphorylation (121, 122), on the analogy with other biological enzymatic cascade pathways like, e. i. the *Complement* that is upstream controlled by the C1q-inhibitor (C1qINH).
A disruption of the mechanisms regulating the NFkappaB and IkappaB balance has been proposed as related to the development of many immune mediate inflammatory diseases (123).
The Toll-like receptors (TLRs) belong to a type I integral membrane receptors recognizing pathogen associated molecular patterns (PAMPs) that are conserved microbe derived molecules.
Diverse TLRs can recognize diverse molecules from microbial cell-wall or can recognize viral RNA and bacterial DNA and thus, despite invariant, constitute a restricted repertoire of diversity recognition.
If mainly investigated for their paramount role in the activation of innate immunity and, as emerging, as critical controller of the acquired immunity where different TLRs can have different effects, TLRs are broadly distributed on a variety of tissue. Particular interest is emerging in studies about epithelial TLRs distribution and function that together with NF-kappaB defect could be of some importance in the discussion of our data in the contest of celiac disease.
Among the many functions, NF-KappaB exerts an active control on apoptotic balance; very recently it has been reported that NF-kB deficiency leads to apoptosis of colonic epithelial cells and to the development of a chronic inflammatory response, involving at the beginning cells of the innate immunity and later T-cells, similar to the histopathology of inflammatory bowel diseases (124).
Another emerging mechanism involved into colonic homeostasis appears linked to the anatomical location of Toll-like receptors with a special role for TLR9.
Among the several TLRs expressed on the cell surfaces of intestinal epithelium, TLR9 appears to deliver inhibitory signal on NF-kappaB when located at the cell apex whereas activating signal if basolaterally located (125-127).

Data discussion

Hematopoietic Precursor and stem cell CD34+ circulating in the peripheral blood of patients suffering from celiac disease resulted highly and significantly ($p< 0.0001$) increased with respect to healthy population.
Such a feature has been well investigated in allergic diseases (25-42), particularly in asthma, also as a consequence of allergen challenge and production of stromal-cell derived factor 1 alpha (SDF-1 alpha) that is capable of inducing a bone marrow (BM) CD34+ mobilization (39) or to investigate mechanisms underlying bronchial remodelling (128) or other conditions, such as heart stroke in which CD34+ HPC are involved in post-lesion reparative fibrosis (129) or during gut epithelial reparative processes (61, 62). Nevertheless, as myofibroblasts move just at the beginning of asthma inflammation, at the mild intermittent step, and thus appear to induce fibrosis without anatomical injuries, also in CD patients the increased

traffic of CD34+ HPC resulted not significantly related ($p = 0.41$) with intestinal epithelial damage as assessed by biopsy, underscoring an interpretation as a possible ongoing reparative mechanism.

A discussion related to the emerging function of the gut as a primary immune organ, as suggested by the compelling evidence briefly reviewed, is intriguing not only for CD pathogenetic implications but also for the aspect potential regarding the peripheral tolerance failure.

In fact, if central tolerance is a matter for the Thymus, peripheral immune homeostasis, particularly on adulthood after thymus involution, appears to involve gut associated lymphoid tissue more and more.

Furthermore, a better knowledge of the molecular signalling pathways that regulate the development of different cell subpopulations with different activities on the delicate equilibrium between anergy-tolerance and action sustaining the immune homeostasis may allow a view more consistent of some clinical observations previously considered paradoxical.

From the above mentioned evidence two fundamental elements rise: the first is in that *tolerance/hyporesponsiveness* appears a function proper of the *immature* cells belonging to a committed lineage when the acquisition of *effector* functions is a consequence of the transcription of the differentiation program along the lineage.

The second, that could explain the why of the reported increased traffic of CD34+HPC, is the mechanism that maintains anergy or allow the progression towards the effector stages that is based on cell death.

Apoptotic balance is under the active control of NfkappaB, as reported (119-124), that activated on demand, down-regulates cellular death allowing survival programs required for the differentiation.

Such a model suggests that on resting condition hyporesponsiveness/anergy is maintained by a controlled-cell death of the immature compartment (H2) that thus requires a constant incoming of immature precursor cells necessary to replace the programmed loss.

Under physiological conditions the HPC supply may be guaranteed by the local reservoirs that are niches and crypto-patches without any bone-marrow contribution (CD34+ HPC peripheral circulation in normal subjects is very low if absent); a defect afflicting NFkappaB/IkappaB activity, as reported (123, 124), may results in an excessive high-rate of apoptosis that induces an HPC extra-demand that requires bone-marrow mobilization and the observed increased peripheral traffic of CD34+ HPC (Fig. 3).

A quite constant pattern skewed to TH2 conditions, observed at the circulating cell compartment, also in TH1 locally dominated diseases, could be consistent with the emerging scenario.

Celiac disease could represent a perfect model where an expansion of the immature-cell compartment sustains a final TH1/TH17, antigen-specific (gliadin), autoimmune reaction.

Our data, in fact, show an overwhelming H2-oriented lymphoid pattern in the peripheral blood and, even in the three cases where H1 pattern prevailed, the CD34+ values were very high.

A coexistence between TH2 and TH1 behaviours (130-132) could be thus explained on the model that physiological response is TH2 and becomes TH1 when survival programs switched on by extrinsic stimuli allow the differentiation progress; under not-physiological conditions an expanded H2 compartment may receive persistent survival signals by extrinsic antigens such as gliadin. Thus, anatomically, we could identify H2 bearing markers cells at the crypto-patches level where differentiating processes involve CD34+ precursors and stem cells and H1 cells at the villous level, with intermediate patterns of cytokine secretion (IL10, TGF-beta) detectable along the differentiation/maturation pathway.

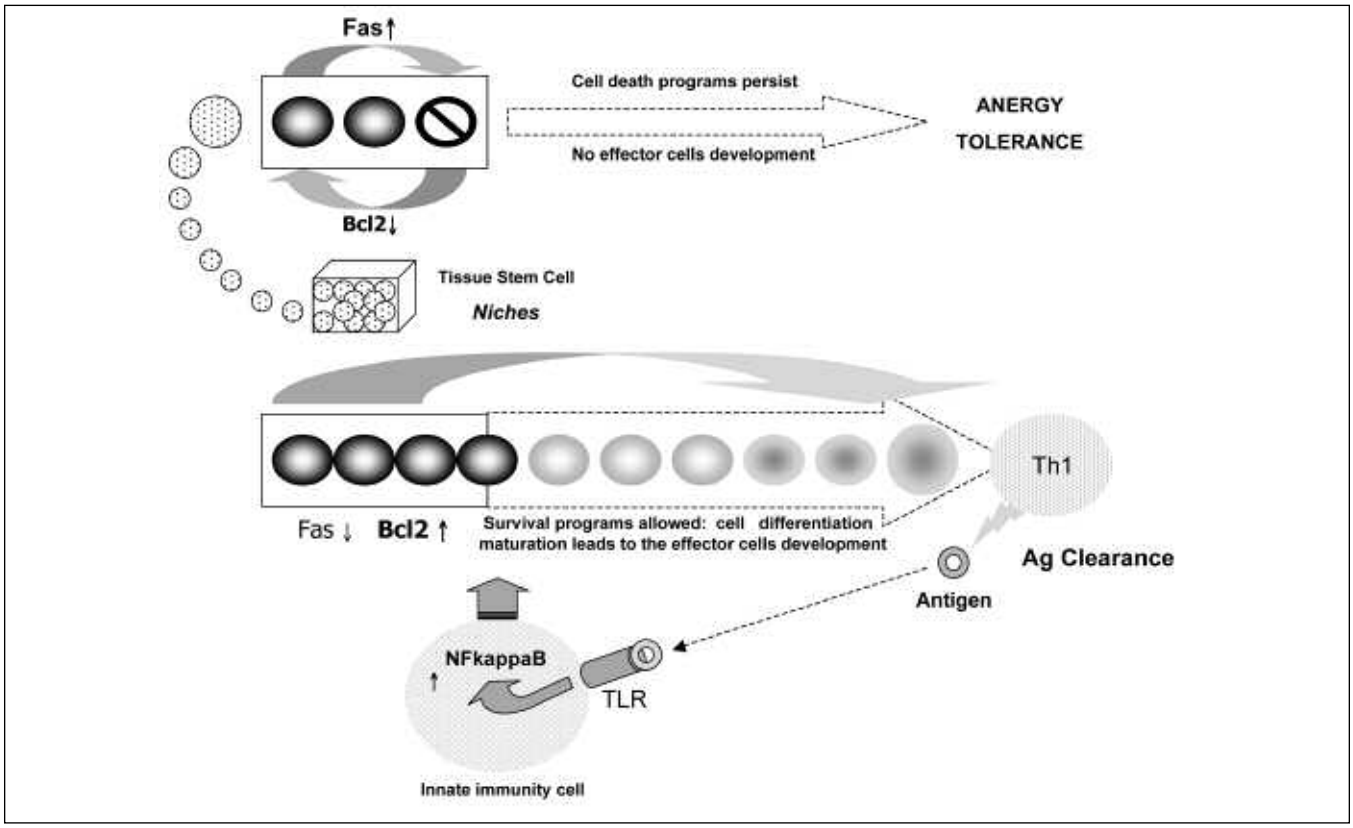
Among the regulatory cell populations if poor evidence exists for a Tregs defect in CD (133), a large body of experimental data suggests a disturbed homeostasis of the lymphoid NK subsets to play a central role not only in celiac disease but also in the determinism potential of many of the other chronic inflammatory frequently associated diseases.

The acquisition that different suppressor/stimulating properties depend on the progression of the cell differentiation/maturation pathway has been well defined for NK-lineages as well as defects of the regulatory systems are going to be clarified.

Such studies evidenced as developmental stages close to lineage-precursors (immature cells) display suppressor/anergic function when modulating properties are acquired at the intermediate stages to become effector cells at the ultimate steps of the maturational process; the H2 – H0 – H1 linear version, originally developed for educational purposes, results useful to describe the pattern of cytokine and chemokine associated with the different developmental/functional status.

Two elements emerged as critically controllers of the differentiation progress from lineage precursors onwards: IL12 and NFkappaB (101-120).

Figure 3 - Emerging potential model of lymphoid lineages homeostasis, as suggested by recent evidence. In absence of antigenic stimuli, immature cells can not differentiate because subjected to apoptotic programs that guarantee hypo-responsiveness/anergy. Under physiological conditions, lost cells are replaced by precursors locally harboured in the tissue *niches* (upper section) and thus CD34+ traffic in peripheral blood is very low if absent. When an antigen has to be removed, it is firstly engaged by toll-receptors of cells belonging to innate immunity with a consequent activation of NFkappaB. Apoptosis is then down-regulated and survival programs allow the proceeding of the maturational events leading to the differentiation of effector Ag-specific Th1 cells. NFkappaB deficiency thus, could reduce the effector Th1 cell development resulting in a immunodeficiency. Permanent up-regulation of apoptotic programs, furthermore, may induce an excessive cell death that can not be replaced by the exhausted local *niches* requiring an extra-supply of bone-marrow derived circulating precursor and stem cells



Since it is recognized that IL12 gene is under NFkappaB control, a defect of the latter appears capable of disregulating the entire lineage ontogeny from the beginning.

A deficiency of NFkappaB activity in fact, is necessary not only for the repression of apoptosis and the allowing of survival programs but also for the transcription of IL12, essential for the expression of the receptor regulatory family CD94/NKG2 A-G.

We have previously stressed the attention on the reported deficiency in inflammatory bowel diseases of a particular invariant NKT cell population (iNKT) (111, 112).

The main features of such iNKT subset are suppressive action, an early developmental status close to the precursors (116) and the expression of a truncated-like receptor (pre-TcR) using the Vbeta 8.2 chain not rearranged but germline encoded (116, 117).

The reported deficiency of a lymphoid subset Vbeta 8.2+ also in active allergic respiratory disease (115) could suggest that such deficiency may represent a defect shared by respiratory and digestive immune-mediated inflammatory diseases. Since the Vbeta 8.2+ deficiency was absent in an allergic population asymptomatic after an effective regimen of low-doses allergen-specific sublingual immunotherapy, a low-dose gliadin SLIT expanding gliadin-specific Tr1 IL10-producing-cells may represent the most convenient approach to restore gliadin tolerance (134) with respect to other experimental approach of oral tolerance induction based on the ad-

ministration of adjuvant-coupled antigen, expanding FoxP3+ Tregs (135).

A NFkappaB deficiency, furthermore, not only induces an exaggerated apoptosis rate of the lymphoid immature cell compartment but, equally, of the intestinal epithelial cells (124) and it is conceivable that the anatomo-physiological sources of precursors necessary to the normal-rated cellular turnover, that are crypto-patches, are incapable of supporting the excessive-rated cellular death leading to the epithelial progressive destruction.

Auto-antibodies of A class to transglutaminase, that are the only parameter significantly correlated (p= 0.02) with histological damage, could furthermore worsen NFkappaB deficiency. Tranglutaminase, in fact, has been reported to enhance NFkappaB activity by the polymerisation of the inhibitor IkappaB (136).

Celiac disease, thus, represents an experiment of nature from which many lessons could be learned.

It has a component depending on Th1-antigen specific reaction that characterise the inflammation at the villous and that is well controlled by a strict gluten-free diet; furthermore NFkappaB deficiency may induce an abnormal death rate of cell belonging to the immature compartment where evidence suggests to reside regulatory subsets.

Such a regulatory populations deficiency could affect not only the local gliadin-specific reaction but also peripheral tolerance, mainly in the adulthood when thymus regulatory activity declines.

A reduction of suppressor cells systemic activity, particularly of the NKT-lineage, whose physiological ontogeny appears linked to the GALT function as primary lymphoid tissue, could thus underlie the frequent association of CD with allergic and autoimmune diseases.

Under this optic, the highly significant (p< 0.0001) increased peripheral traffic of CD34+ precursor and stem cells appears more related to a compensatory mechanism consequent to the excessive cell-death rate due to the NFkappaB malfunction rather than to the gluten-specific Th1 reaction itself.

In other words, an increased input of CD34+ HPC in a gut where the anatomical segregation of the immature cells into crypto-patches is disrupted because of the compromised epithelial barrier, may result in an activation of such immature cells by the polyclonal activators largely represented in common foods, raising the question as to whether in Refractory Celiac Disease (137) a diet free only of gluten could be still regarded as adequate.

Concluding, meanwhile protocols devoted to expand gluten-specific regulatory-cells will be developed as well as synthetic agonists of the diverse Toll-like receptors will be better investigated (138, 139), an attempt to limit NFkappaB inefficiency by means of the stimulation of different TLRs using probiotical formula containing more than one bacterial strain may be considered.

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