University of Crete

School of Medicine

The Molecular Basis of Human Diseases

Master Thesis

Association of T-regulatory cells (Tregs) with the subpopulations of Myeloid-Derived Suppressor Cells (MDSCs) in patients with Chronic Idiopathic Neutropenia (CIN)

Συσχέτιση των Τ ρυθμιστικών κυττάρων (T regulatory cells, Tregs) με τους υποπληθυσμούς των Μυελικών Κατασταλτικών Κυττάρων (Myeloid-Derived Suppressor Cells, MDSCs) σε ασθενείς με Χρόνια Ιδιοπαθή Ουδετεροπενία (Chronic Idiopathic Neutropenia, CIN)

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Contents

Περίληψη 3

Abstract 4

Introduction 6

1) **Chronic idiopathic neutropenia (CIN)** 6
2) **Myeloid Derived Suppressor Cells (MDSCs)** 7
3) **Regulatory T-cells (Tregs)** 11

Hypothesis – Aim of the study 18

Materials and methods 18

1) **Patients and Controls** 18
2) **Peripheral Blood Mononuclear Cells (PBMC) and Bone Marrow Mononuclear Cells (BMMCs) isolation** 19
3) **Immunophenotyping - Cell staining** 19
4) **Flow cytometry principals** 21
5) **Functional studies: T-Cell suppression assay** 23
6) **Proliferation analysis** 28
7) **Statistical analysis** 29

Results 29

1) **Cumulative data** 29
2) **M-MDSCs & PMN-MDSCs in PB & BM of CIN patients & healthy controls** 31
3) **Tregs in PB & BM of CIN patients & healthy controls** 35
4) **Tregs vs MDSCs in patients** 42
5) **MDSCs capacity to suppress T-cell proliferation** 45

Conclusions 46

References 48

Περίληψη

Η χρόνια ιδιοπαθή ουδετεροπενία (CIN) είναι μια διαταραχή των ουδετερόφιλων που χαρακτηρίζεται από παρατεταμένη και ανεξήγητη μείωση του αριθμού των ουδετερόφιλων περιφερικού αίματος (PB) κάτω από το κατώτερο όριο του φυσιολογικού εύρους για παρατεταμένη χρονική περίοδο (περισσότερο από 3 μήνες). Ο κύριος παθογενετικός μηχανισμός της CIN περιλαμβάνει αυξημένη απόπτωση, που προκαλείται από Fas, των $CD34^{+}CD33^{+}$ μυελικών προγονικών κυττάρων. Στην CIN συμμετέχει επίσης χρόνια φλεγμονή που προκαλείται από ένα ανασταλτικό μικροπεριβάλλον μυελού των οστών (BM) που αποτελείται από ενεργοποιημένα Τ-λεμφοκύτταρα (ολιγοκλωνικό προφίλ) και προφλεγμονώδεις μεσολαβητές (TNF-α, TGF-β1, Fas-συνδέτης, IFN-γ, IL-1b και IL-6).

Τα Μυελικά Κατασταλτικά Κύτταρα (MDSCs) είναι ανώριμα κύτταρα μυελικής προέλευσης, χωρισμένα σε δύο υποπληθυσμούς, τα πολυμορφοπυρηνικά (PMN)-MDSCs και τα μονοκυτταρικά (M)-MDSCs. Τα MDSCs έχουν αναδειχθεί ως σημαντικοί συντελεστές της επέκτασης του όγκου και της χρόνιας εξέλιξης της φλεγμονής προκαλώντας ανοσοκατασταλτικούς μηχανισμούς, αγγειογένεση και αντοχή στα φάρμακα. Μέσω της ενεργοποίησης των ενζύμων αργινάση 1 (Arg1) και συνθετάσης νιτρικού οξειδίου 2 (NOS2) και της παραγωγής αντιδραστικών ριζών οξυγόνου (ROS), οδηγούν σε καταστολή του πολλαπλασιασμού των Τ-κυττάρων, αναστολή της κυτταροτοξικότητας των Natural Killer (NK) κυττάρων, διαμόρφωση της πόλωσης των μακροφάγων και επαγωγή ανάπτυξης ρυθμιστικών Τ-κυττάρων (Tregs).

Τα Tregs έχουν περιγραφεί καλά τα τελευταία χρόνια ως ένα ξεχωριστό υποσύνολο των Τ-κυττάρων που είναι τόσο αναπτυξιακά όσο και λειτουργικά μοναδικά και απαραίτητα για τη διατήρηση της ανοσοποιητικής ομοιόστασης και της αυτο-ανοχής. Τα Tregs καταστέλλουν τη λειτουργία άλλων Τ-κυττάρων για να περιορίσουν την ανοσο-απόκριση. Οι κύριοι υποπληθυσμοί κυττάρων Treg περιλαμβάνουν φυσικά κύτταρα Treg (nTreg) που παράγονται στον θύμο αδένα και επαγόμενα κύτταρα Treg (iTreg) που παράγονται στην περιφέρεια από συμβατικά FoxP3-CD4+ Τ-κύτταρα. Τροποποιήσεις στον αριθμό και τη λειτουργία των Tregs έχουν εμπλακεί σε διάφορες αυτοάνοσες ασθένειες. Επίσης, έχουν βρεθεί υψηλά επίπεδα Tregs σε πολλές κακοήθεις διαταραχές. Τα Tregs μπορούν επίσης να αποτρέψουν τις αντικαρκινικές ανοσο-αποκρίσεις, οδηγώντας σε αυξημένη θνησιμότητα.

Με βάση προηγούμενες μελέτες από το εργαστήριό μας γνωρίζουμε ήδη ότι οι ασθενείς με CIN εμφανίζουν χαμηλό ποσοστό MDSCs στο PB (περιφερικό αίμα) και στον BM (μυελό των οστών), σε σύγκριση με υγιή άτομα. Επίσης, οι ασθενείς με CIN εμφανίζουν υψηλότερη συσσώρευση PMN-MDSC στον BM (έναντι του PB) σε σύγκριση με υγιή άτομα. Τέλος, γνωρίζουμε ότι τα MDSCs στη CIN εμφανίζουν φυσιολογική ικανότητα καταστολής των Τ-κυττάρων. Έτσι, υποθέσαμε ότι τα χαμηλά ποσοστά των υπο-πληθυσμών των MDSCs μπορεί να έχουν ρόλο στην επιμονή των φλεγμονωδών διεργασιών που σχετίζονται με τη CIN, και η συσσώρευση των PMN-MDSCs στον BM μπορεί να αντιπροσωπεύει έναν αντισταθμιστικό μηχανισμό για την καταστολή των φλεγμονωδών διεργασιών στον BM των ασθενών.

Επιπλέον, υποθέσαμε ότι τα Tregs μπορεί επίσης να είναι επηρεασμένα σε αριθμό και λειτουργικότητα σε ασθενείς με CIN, και συνεπώς να συμβάλλουν στην

παρατεταμένη φλεγμονώδη διαδικασία στον ΒΜ. Τέλος, υποθέσαμε ότι τα MDSCs ενδέχεται να έχουν εξασθενημένη / αλλοιωμένη ιδιότητα να επάγουν τον πληθυσμό των Tregs σε ασθενείς με CIN. Για να το διερευνήσουμε αυτό, πραγματοποιήσαμε μια σειρά πειραμάτων που περιελάμβαναν 1) τον ποσοτικό προσδιορισμό α) των MDSCs σε PB ασθενών CIN έναντι υγιών ατόμων, β) των MDSCs σε PB έναντι ΒΜ ασθενών CIN, γ) των Tregs σε ασθενείς CIN έναντι υγιή άτομα, με κυτταρομετρία ροής, και 2) τη συσχέτιση των MDSCs και των Tregs. Τέλος, η ανοσοκατασταλτική λειτουργία των MDSC μελετήθηκε μέσω του προσδιορισμού της καταστολής του πολλαπλασιασμού των Τ-κυττάρων σε συνθήκες με και χωρίς την παρουσία τους.

Η μελέτη αποκάλυψε ότι τα MDSCs είναι χαμηλότερα σε ασθενείς με CIN σε σύγκριση με τα φυσιολογικά, ενώ τα Tregs είναι υψηλότερα σε ασθενείς σε σύγκριση με τα φυσιολογικά. Επίσης, τα CIN MDSC εμφανίζουν φυσιολογική ικανότητα να καταστέλλουν τον πολλαπλασιασμό των Τ-κυττάρων in vitro. Ο αριθμός των CD25high-high Tregs συσχετίστηκε με τον αριθμό των PMN-MDSCs. Έτσι, καταλήξαμε στο συμπέρασμα ότι δεν υπάρχουν ενδείξεις ότι τα CIN MDSCs εμφανίζουν μειωμένη ικανότητα να επάγουν τα Tregs. Αντίθετα, μπορεί να υποτεθεί ότι τα PMN-MDSCs μπορεί να έχουν ρόλο στην in vivo επαγωγή των Tregs στους ασθενείς μας, καθώς και ότι αυτά τα κύτταρα μπορεί να προκαλέσουν φυσιολογικό πολλαπλασιασμό των Tregs. Προφανώς, η παραγωγή MDSCs επηρεάζεται / μπλοκάρεται στη CIN με διαφορετικό τρόπο από την παραγωγή των Tregs, που φαίνεται να είναι φυσιολογική. Τα Tregs επάγονται από διάφορα μονοπάτια εκτός από τα MDSCs. Τα Tregs μπορεί να αυξάνοναται ίσως ως αντισταθμιστικός μηχανισμός για την καταστολή της φλεγμονώδους διαδικασίας στον ΒΜ των ασθενών και ως αντισταθμιστικός μηχανισμός για την μειωμένη παραγωγή MDSCs.

Abstract

Chronic idiopathic neutropenia (CIN) is a neutrophil disorder characterized by prolonged and unexplained reduction in the number of peripheral blood (PB) neutrophils below the lower limit of the normal range for a prolonged period (more than 3 months). The main pathogenetic mechanism of CIN implicates increased Fas-mediated apoptosis of CD34+CD33+ myeloid progenitor cells. Chronic inflammation driven by an inhibitory bone marrow (BM) microenvironment consisting of activated T-lymphocytes (oligoclonal profile) and proinflammatory mediators (TNF-α, TGF-β1, Fas-ligand, IFN-γ, IL-1b, and IL-6) is also involved.

Myeloid Derived Suppressor Cells (MDSCs) are immature cells of myeloid origin, divided into two subpopulations, the polymorphonuclear (PMN)-MDSCs and the monocytic (M)-MDSCs. MDSCs have emerged as important contributors of tumor expansion and chronic inflammation progression by inducing immunosuppressive mechanisms, angiogenesis and drug resistance. Through activation of the enzymes arginase 1 (Arg1) and nitric oxide synthase 2 (NOS2), and production of reactive oxygen species (ROS), they lead to suppression of T-cell proliferation, inhibition of natural killer (NK) cell cytotoxicity, modulation of macrophage polarization and induction of development of regulatory T-cells (Tregs).

Tregs have been well described over the past several years as a distinct subset of T cells that are both developmentally and functionally unique and essential to maintaining immune homeostasis and self-tolerance. Tregs suppress the function of other T-cells to limit the immune response. The major subpopulations of Treg cells include natural Treg (nTreg) cells that are produced in the thymus and induced Treg (iTreg) cells that are generated in the periphery from conventional FoxP3-CD4+ T-cells. Alterations in the number and function of Tregs have been implicated in several autoimmune diseases. Also, high levels of Tregs have been found in many malignant disorders. Tregs may also prevent antitumor immune responses, leading to increased mortality.

Based on previous studies from our laboratory we already know that CIN patients display low proportion of MDSCs in the PB (peripheral blood) and BM (bone marrow), compared to healthy individuals. Also, CIN patients display higher PMN-MDSC accumulation in BM (vs PB) compared to healthy individuals. Finally, we know that MDSCs in CIN display normal T-cell suppression capacity. So, we assumed that the low proportions of MDSC subsets may have a role in the persistence of the inflammatory processes associated with CIN, and the accumulation of PMN-MDSCs in the BM may represent a compensatory mechanism to suppress the inflammatory processes within patients' BM.

Moreover, we assumed that Tregs may also be altered in number and functionality in patients with CIN, and thus contribute in the sustained inflammatory process in the BM. Finally, we hypothesized that MDSCs may have impaired/altered property of inducing the Treg population in patients with CIN. In order to investigate this, we performed a series of experiments that included 1) the quantification of a) MDSCs in PB of CIN patients vs healthy individuals, b) MDSCs in PB vs BM of CIN patients, c) Tregs in CIN patients vs healthy individuals, by flow cytometry, and 2) the correlation of MDSCs and Tregs. Finally, the immunosuppressive function of MDSCs was studied by T-cell suppression assays in conditions with and without their presence.

The study revealed that MDSCs are lower in CIN patients compared to normal, but Tregs are higher in patients compared to normal. Also, CIN MDSCs display normal capacity to suppress Tcell proliferation in vitro. The number of CD25high-high Tregs correlated with the number of PMN-MDSCs. Thus, we concluded that there is no evidence that CIN MDSCs display impaired capacity to induce Tregs. In contrary, a role for PMN-MDSCs in the in vivo expansion of Tregs in our patients may be assumed, as these cells may induce normally Treg proliferation. Obviously, the production of MDSCs is impaired/blocked in CIN in a different manner than the production of Tregs, which is normal. Tregs are induced by several pathways besides MDSCs, and are elevated maybe as a compensatory mechanism to suppress the inflammatory process within patients' BM and as a compensatory mechanism to their impaired production of MDSCs.

Introduction

1) Chronic idiopathic neutropenia (CIN)

Chronic idiopathic neutropenia (CIN) is a neutrophil disorder characterized by prolonged and unexplained reduction in the number of peripheral blood (PB) neutrophils below the lower limit of the normal range, for a prolonged period (more than 3 months) in adults (1).

Depending on the number of neutrophils in the blood, the following three categories of neutropenia can be distinguished:

- ✓ ‘’mild’’, if the number of neutrophils ranges from 1800 to 2499 per ml of blood,
- ✓ ‘’moderate’’, if the number of neutrophils ranges from 500 to 1799 per ml of blood and
- ✓ ‘’severe’’ if the number of neutrophils is below 500 per ml of blood.

The diagnostic criteria established for CIN are:

- ✓ Number of circulating neutrophils below 2500 per ml of blood found at least in four consecutive blood cell counts performed monthly within the last three months of observation.
- ✓ Exclusion of cyclic and familial neutropenia by appropriate studies.
- ✓ No evidence of any underlying disease associated with neutropenia following a detailed hematological, biochemical, virologic, cytogenetic, immunologic, parasitologic, and ultrasonic investigation.
- ✓ No previous exposure to ionizing irradiation, contact with organic solvents or intake of cytotoxic or other drugs capable of causing neutropenia.
- ✓ Negative leukoagglutination and immunofluorescent tests for anti-neutrophilic antibodies (2).

Extensive research on neutropenia has been performed in the Hematopoiesis research laboratory at University of Crete. The etiology of CIN is not entirely known. However, there is evidence suggesting that the pathophysiology of CIN is related to an inflammatory Bone Marrow (BM) microenvironment consisting of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukin-1β (IL-1β), proapoptotic mediators such as Fas-ligand (FAS-L), and activated oligoclonal or monoclonal T lymphocytes with myelosuppressive properties, which collectively induce the accelerated apoptotic death of the granulocytic progenitor cells (3–5). Increased levels of proinflammatory cytokines are also found in the PB of CIN patients. The cellular origin of these cytokines in the BM and PB of CIN patients remains largely unknown (6).

Generally, CIN patients do not need any treatment. However, in the cases of severe neutropenia and infection they may need to be treated with antibiotics and granulocyte colony stimulating factor (G-CSF), which increases rapidly the number of circulating neutrophils. Splenomegaly, osteoarthritis and osteoporosis are conditions more common in patients with CIN than the general population. The disorder is thought to be by some researchers a preleukemic state since cases of patients with

CIN developing acute myeloid leukemia have been reported in some centers including ours (5,7–11).

2) Myeloid Derived Suppressor Cells (MDSCs)

Characteristics and Function

Myeloid Derived Suppressor Cells (MDSCs) are immature cells of myeloid origin with immunosuppressive properties that accumulate during chronic inflammation and tumor progression. MDSCs are divided into two main subpopulations, the polymorphonuclear (PMN)-MDSCs and the monocytic (M)-MDSCs, characterized by the immunophenotype HLA-DRlow/-CD11b+CD33+CD15+ and HLA-DRlow/-CD11b+CD33+CD14+, respectively (12). However, it should be noted that there is another subset of MDSCs with the Lin(CD3CD14CD15CD19CD56)-HLA-DR-CD33+ immunophenotype comprising of more immature progenitors. These cells are called early-stage MDSCs (e-MDSCs) (13).

MDSCs derive from the hematopoietic BM precursor cells and acquire strong immunosuppressive and tumorigenic activities. These functions include: the elimination of essential amino acids for T cell proliferation, such as arginine and cysteine; the production of nitric oxide (NO) and reactive oxygen species (ROS) that causes the nitration of T cell receptors (TCR) (12); the production of indoleamine-2,3-dioxygenase (IDO), an enzyme that catalyzes the first and rate-limiting step in the kynurenine pathway, causing depletion of tryptophan and production of the catabolic product kynurenine, which can slow the growth of T cells, impair their survival and promote T-regulatory cells (T-regs) generation (14); the production of chemokines responsible for T cell migration and T cells and Natural Killer (NK) cells apoptosis; the production of IL-10 and transforming growth factor (TGF)-1, which both inhibit T-cell functions and induce T-regs activation; the expression of programmed death-ligand 1 (PD-L1), which can interact with PD-1 receptor expressed on T cells and reduce their activity; the reduction of the TCR-chain expression, which makes TCR-mediated antigen recognition more difficult; the secretion of angiogenic factors promoting tumor neo-vascularization; and finally the production of growth factors, matrix metalloproteinases and cytokines that can stimulate tumor growth and activate Tregs (12). (Figure 1)

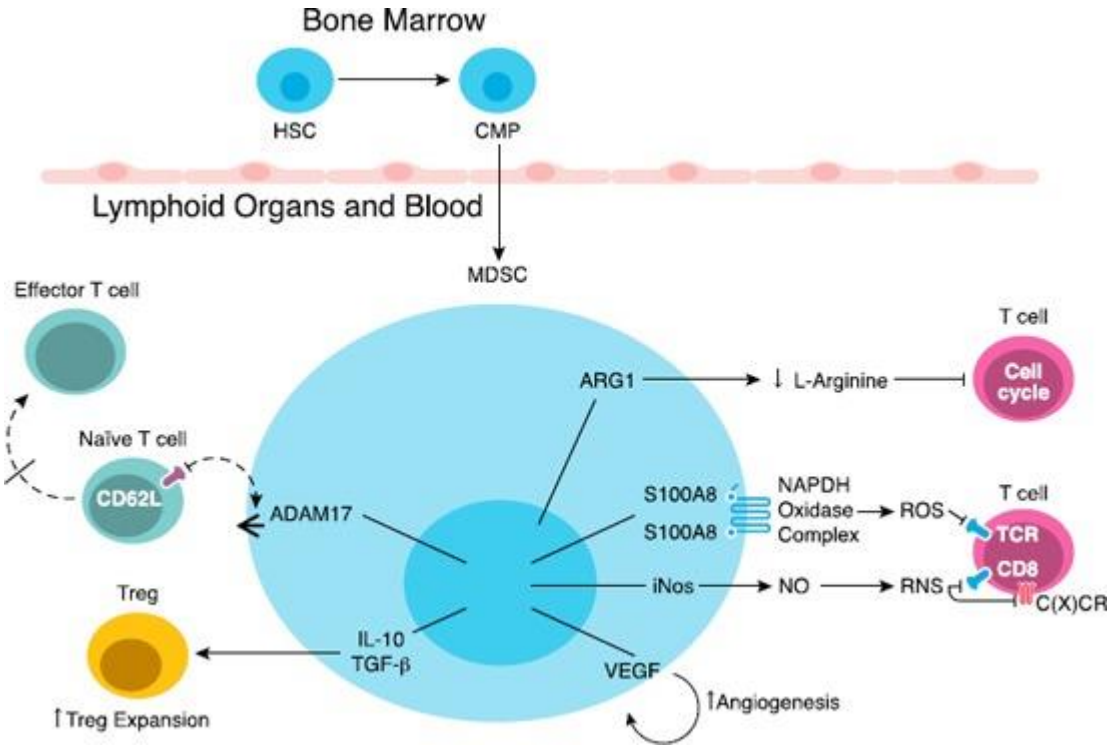


Figure 1: Overview of MDSC immunosuppressive mechanisms

Under steady-state conditions, hematopoietic stem cells (HSCs) located in the bone marrow give rise to common myeloid precursors (CMPs), which then differentiate into mature myeloid cells. During tumor progression, CMPs give rise to MDSCs, which subsequently accumulate in blood and in lymphoid organs, such as the spleen. Immunosuppressive MDSCs suppress the immune system by distinct mechanisms, including induction of T-regs proliferation; production of high levels of arginase 1 (ARG1) that depletes T cells of L-arginine; production of high levels of ROS and nitrogen species (RNS; peroxynitrate) that lead to nitration and nitrosylation of TCR, CD8, and chemokine C(X)CRs receptors; promotion of angiogenesis; and blockage of the migration of naive CD62L+ T cells to lymphoid organs, which results in diminished expansion of effector T cells ADAM17, ADAM disintegrin, and metallopeptidase domain 17 and S100A8 and S100A9 calcium-binding proteins. (Chesney, Mitchell, and Yaddanapudi 2017).

Generation, Expansion and Activation

MDSCs are generated from the pathologic modulation of myelopoiesis that is induced by constantly produced inflammatory mediators under chronic inflammation in the tumor microenvironment. The generation and expansion of MDSCs is a complex process that requires the presence of two major signals. The first is responsible for the production of immature myeloid cells with no terminal differentiation and the second is responsible for their immunosuppressive function (13). (Figure 2) The first group of signals is mostly driven by tumor-derived growth factors. Tumor cells are able to produce a variety of growth factors and inflammatory mediators including granulocyte

macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), stem cell factor (SCF), vascular endothelial growth factor (VEGF), TGF, TNF, IL-1, IL-6, and IL-10 that induce MDSCs production. In addition, various chemokines such as CCL2, CCL3, CCL4, CCL5, CXCL1 and CXCL8 are responsible for the migration of the previously generated MDSCs into the tumor site where the second group of signals takes place (12). The second group of signals is mostly driven by the tumor microenvironment and tumor stromal cells that produce inflammatory cytokines such as IFN-γ, IL-1β, IL-4, IL13, TNF-β, toll-like receptor (TLR) ligands, PGE2 (12) and is mediated by transcription factors STAT1, STAT6 and nuclear factor (NF)-κB as well as by elevation of cyclooxygenase (COX)-2 activity (15). Finally, Bruton's tyrosine kinase (BTK), a key component of B-cell receptor signaling and B-cell development has recently been found to be highly expressed by MDSCs, revealing its role in the generation, maturation, trafficking and function of these myeloid cells (16). All factors mentioned above are able to stimulate MDSCs generation, enrichment and activation leading to the inhibition of T-cell functions, the antitumor reactivity of dendritic cells (DCs) and NK cells and the stimulation of T-regs (12).

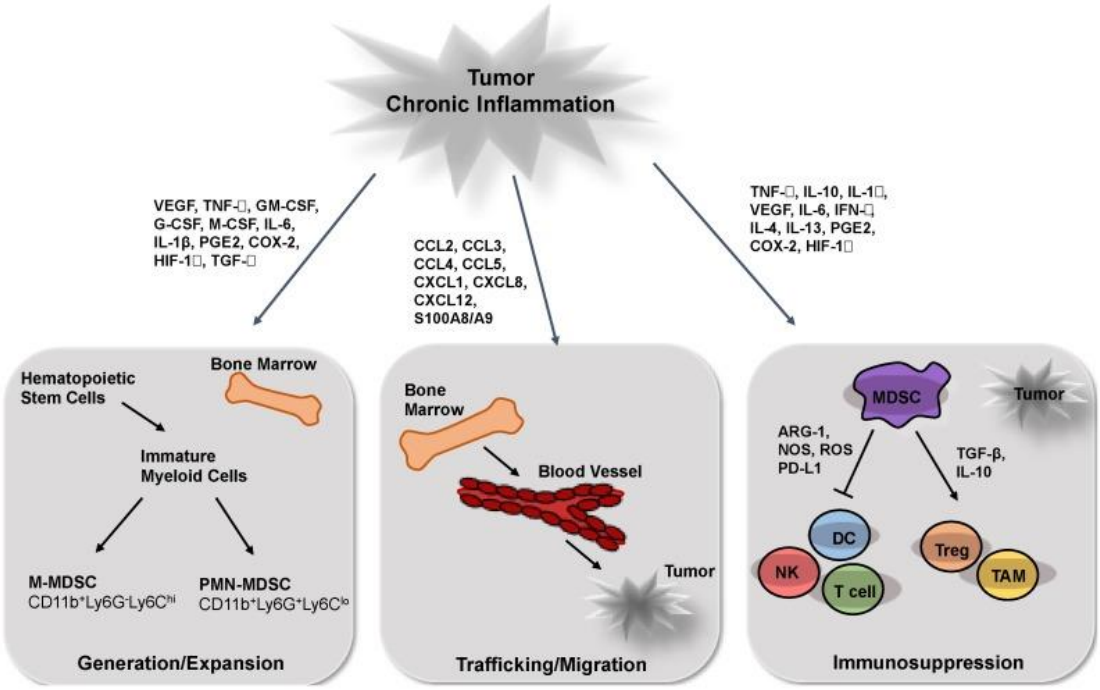


Figure 2: Chronic inflammatory factors that MDSCs generation, migration and activation of their immunosuppressive functions at the tumor site. *(Umansky et al. 2016)*

MDSCs stimulate tumor progression

Apart from their immunosuppressive activity, MDSCs enhance tumor progression (Figure 3). MDSCs have been implicated in tumor neo-vascularization and tumor neo-

angiogenesis with the production of VEGF and basic fibroblast growth factor (bFGF). Furthermore, MDSCs have been demonstrated to promote tumor invasion by producing metalloproteinases (MMPs) which enable matrix degradation and chemokines that create a pre-metastatic environment. Metastasis is also promoted by MDSCs through their fusion with tumor cells (12). Finally, S100A8/A9 inflammatory proteins aside from attracting MDSCs into the tumor microenvironment and enhancing their immunosuppressive activity, also promote the activation of mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways in tumor cells, stimulating thereby tumor progression.

MDSCs at the tumor site can also differentiate into tumor associated macrophages (TAMs), which have a tumor-promoting phenotype and can up-regulate the expression of either arginase or iNOS and produce several suppressive cytokines (17). This differentiation from M-MDSCs into TAMs is accompanied by the upregulation of anti-apoptotic molecules FADD-like IL-1β-converting enzyme-inhibitory protein (c-FLIP) and A1, as well as the ARG1 enzyme. TAMs have a relatively high expression of F4/80, intermediate expression of Ly6C, low expression of S100A9 protein and express higher levels of IRF8, a marker of terminal macrophage differentiation compared to M-MDSCs. Together MDSCs and TAMs contribute to non-specific T-cell suppression in the tumor microenvironment (18).

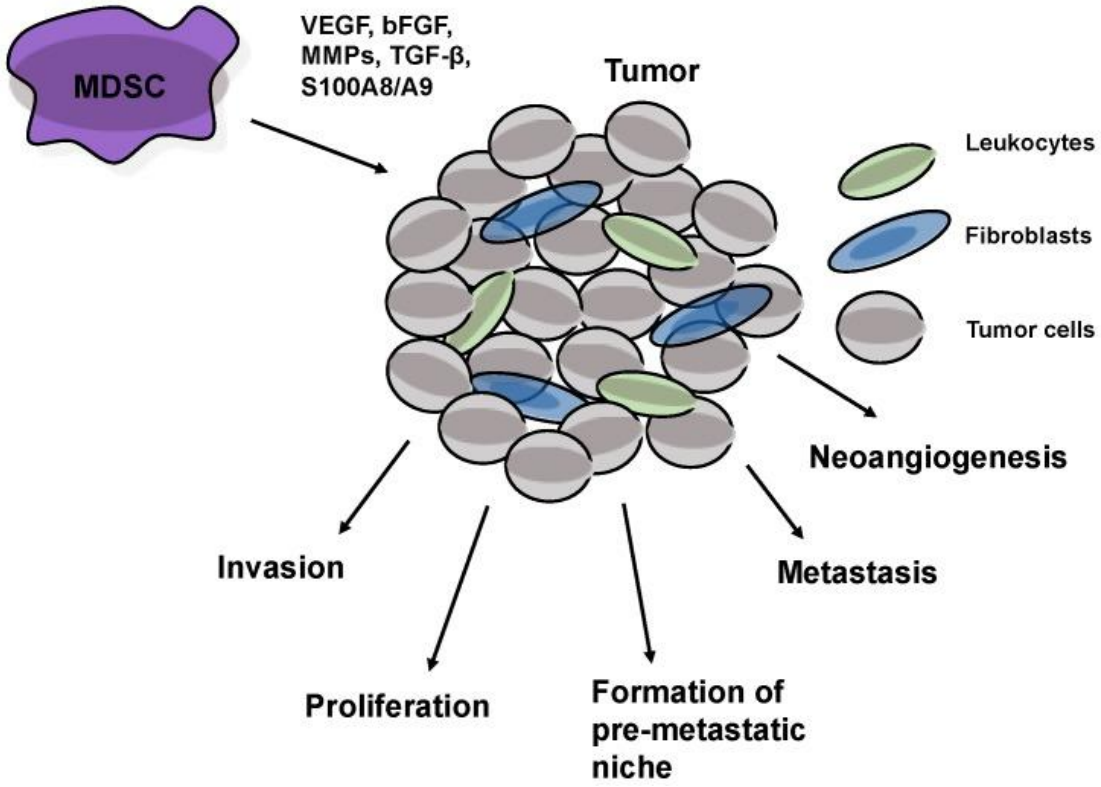


Figure 3: MDSCs support tumor development, metastasis, invasion, proliferation and neo-vascularization through the secretion of MMPs, VEGF, TGF-β, etc. *(Umansky et al. 2016)*

Inhibition of MDSCs immunosuppressive activity

In recent years, MDSCs have been implicated in the resistance to anti-cancer therapies as well as the inhibitory effect of chemotherapy on the immune system. Since MDSCs are cells involved in the inhibition of immune responses and support of tumor progression, their elimination seems to important in order to ameliorate the clinical outcome of cancer patients. Several strategies are currently being tested in order to therapeutically target these cells. These strategies mainly involve the elimination of MDSCs, the deactivation of MDSCs, and the skewing of myelopoiesis away from the generation of MDSCs (19). In more detail, studies have already shown the successful elimination of MDSCs with low doses of chemotherapy. Several trials of testing low dose chemotherapy to increase immune responses have been conducted, and others are still ongoing. In murine models, gemcitabine, has effectively depleted MDSCs populations, resulting in decreased tumor growth and prolonged survival of cancer patients (20). In addition, cis-plastin and 5-fluoro-uracil can eliminate MDSCs and in that way increase CD8+ T-cell responses (21). Moreover, MDSCs can be functionally inactivated by targeting their suppressive properties. ROS and NOS are essential components of MDSC suppressive machinery. NRF2 can modulate the expression of several antioxidant enzymes that scavenge ROS and NO, and its upregulation by a synthetic triterpenoid can downregulate MDSC ROS production (22). To continue with, tadalafil, a phosphodiesterase-5 (PDE-5) inhibitor, has been reported to decrease arginase and iNOS expression by MDSCs and resulted in the upregulation of tumor specific T-cells (23). As for diverting myelopoiesis away from generating MDSCs and into differentiated and mature cells, STAT3 targeting (24), as well as treatment with all-trans-retinoic acid (ATRA) have been shown to drive MDSCs differentiation into dendritic cells (DCs) (25).

Regulatory T-cells (Tregs)

The most important role of the immune response is to protect the body from foreign threats. The immune system must be capable of bringing up an effective immune response against foreign/microbial agents, but must not be self-reactive. This requires the immune system to recognize a countless number of pathogens and retain the ability to differentiate between “self” and “non-self”.

Central tolerance in the thymus is responsible for both generating an extensive repertoire of TCRs through VDJ recombination in T-cells and deleting autoreactive T-cells. Thus, the specificity of the immune response for “non-self” depends on central tolerance, whereby over 95% of the immature T-cells generated in the thymus undergo negative selection to rid the body of autoreactive cells (26).

Besides central tolerance in the thymus though, there are mechanisms in the periphery that abrogate the self-reactive clones that have escaped central tolerance in the thymus. Among these, regulatory T-cells (Tregs) play a critical role in limiting autoimmune and inflammatory processes (27). Tregs, also known as a suppressor T-

cells, belong to a class of white blood cells called lymphocytes. These cells originates from the thymus.

Tregs play important role in maintaining immune homeostasis. Tregs suppress the function of other T-cells to limit the immune response. Alterations in the number and function of Tregs have been implicated in several autoimmune diseases including multiple sclerosis, active rheumatoid arthritis, and type 1 diabetes. High levels of Tregs have been found in many malignant disorders including lung, pancreas, and breast cancers. Tregs may also prevent antitumor immune responses, leading to increased mortality (28).

Two major classes of Tregs have been identified to date: CD4 and CD8 Tregs. CD4 Tregs consist of two types, “natural” Tregs (nTregs) that constitutively express CD25 and FoxP3, and so-called adaptive or inducible Tregs (iTregs). Natural Tregs originate from the thymus as CD4+ cells expressing high levels of CD25 together with the transcription factor (and lineage marker) FoxP3.

nTregs represent approximately 5–10% of the total CD4+ T-cell population, and can first be seen at the single-positive stage of T lymphocyte development. They are positively selected thymocytes with a relatively high avidity for self-antigens. The signal to develop into Tregs is thought to come from interactions between the T-cell receptor and the complex of MHC II with self peptides expressed on the thymic stroma. nTregs are essentially cytokine independent. Adaptive or inducible T-regs originate from the thymus as single-positive CD4 cells. They differentiate into CD25 and FoxP3 expressing Tregs (iTregs) following adequate antigenic stimulation in the presence of cognate antigens and specialized immunoregulatory cytokines such as TGF-β, IL-10, and IL-4. FoxP3 is currently the most accepted marker for Tregs, although there have been reports of small populations of FoxP3- Tregs. The discovery of transcription factor FoxP3 as a marker for Tregs has allowed scientists to better define Treg populations leading to the discovery of additional Treg markers including CD127 (Figure 4) (29–41).

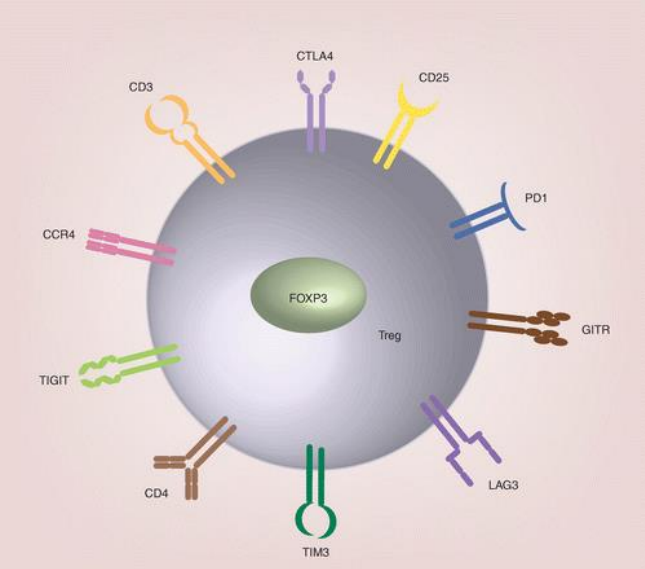


Figure 4: Molecular markers of Tregs *(Pankaj et al. 2019)*

FoxP3: The classic T-reg marker

FoxP3 (also known as Scurfin, IPEX and JM2) is a transcriptional repression factor of the forkhead or winged helix family of transcription factors. FoxP3 has been found to be expressed in all CD4+ Treg cells that have regulatory activity. FoxP3 is useful for confirming purity and yield of isolated Tregs or for characterizing fixed Treg cells (42).

Structure

Human FoxP3 is 47 kDa and the human FoxP3 gene is located on the p arm of chromosome X (Xpll.23). The FoxP3 gene is composed of 11 exons. There are four identifiable domains in the FoxP3 protein. The N-terminal proline-rich domain is involved in suppression of NFkB and Nuclear factor of activated T-cells (NFAT). The leucine zipper domain is required for dimerization or tetramerization. The C-terminal forkhead domain has a nuclear localization sequence and a DNA binding domain (Figure 5). FoxP3 functions to induce the expression of many genes such as CTLA-4, FR4 (folate receptor 4),142 GITR and CD25 and to suppress the expression of other genes such as IL-2, IL-4 and IFN-γ. It has been reported that expression of 700-1000 genes is regulated by FoxP3 either directly or indirectly (42).

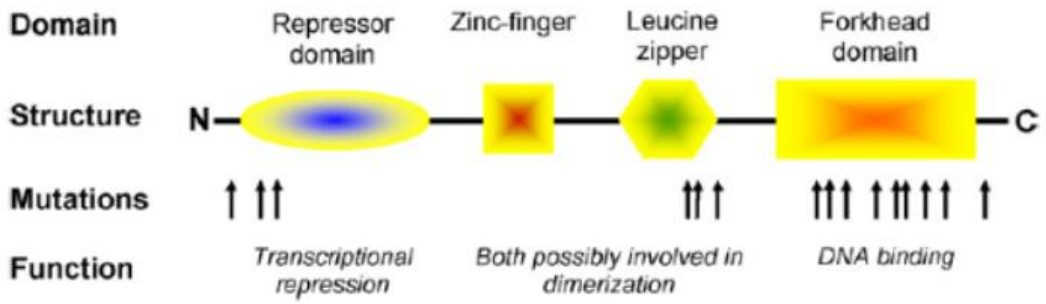


Figure 5: Structure of FoxP3 transcriptional factor

Expression

FoxP3 is highly expressed in lymphoid organs such as the thymus and the spleen. Peripherally, FoxP3 expressing CD4+ T-cells are of the CD4+CD25+ subset (43). While FoxP3 is highly expressed in CD25+CD4+CD8- peripheral T-cells and thymocytes, it is present in low to undetectable levels in both naïve and activated CD4+CD25+ T-cells, as well as in CD4+CD8+ T-, B-, NK-cells. Overall, of the 15% of T-cells expressing FoxP3, 70-80% are also CD4+CD25+. Taken together, the transcription factor FoxP3 is specifically expressed in the CD4+CD25+ population of T-cells, and is required for the generation of regulatory properties.

In the thymus, it develops interaction with Aire+ medullary thymic epithelial cells presenting tissue-specific self-antigens in the context of MHC molecules. The combination of high-affinity TCR interaction with the self-peptide MHC complex along with secondary signals, such as CD28 engagement, direct T-reg lineage commitment in the thymus. CD28 is the main costimulatory molecule in T cells.

CD28 signaling is required for FoxP3 expression and for T-reg cell differentiation program in developing thymocytes (44). These interactions drive a T-reg-specific signaling cascade which upregulates FoxP3 gene expression.

IL-2 plays an important role in T-reg cell development and is critical in maintenance of self-tolerance (45). TGF-β, also, plays key role in the induction and maintenance of immunologic tolerance. TGF-β contributes to the generation, function and survival of adaptive CD4+ and CD8+ T-reg cell subsets (46).

Regulation

Transcriptional regulation of FoxP3 is controlled by factors that target the gene promoter and conserved noncoding sequence 1 (CNS1), 2 (CNS2), and 3 (CNS3). CNS1 contains one enhancer whose activity is regulated by the transcription factors Smad3, NFAT, and AP-1. A second enhancer, located in CNS2, contains highly methylated CpG

sequences in Foxp3− T-cells, but it is demethylated in FoxP3+ Tregs. For this reason, CNS2 was denominated T-reg cell-specific demethylated region (TSDR). Demethylation of the CNS2 region is mediated by the transcription factor Stat5, which critically depends upon IL-2 and TGF-β receptor signaling, thereby illustrating the importance of IL-2 and TGF-β for the development and function of Tregs. Stat5 is a key regulator of chromatin remodeling to make CNS2 region accessible for transcription, whereas AP-1 and Creb exert enhancer 2 activity.

Importantly, CNS2 was shown to be essential for heritable maintenance of FoxP3 expression in dividing Treg cells, with demethylation of TSDR correlating with the stability of FoxP3 expression. Instable FoxP3 expression in iTregs severely limits the usefulness of iTreg adoptive transfer as a cellular therapy for immune undesired reactions (47).

Function

When FoxP3 is expressed following TCR stimulation, it localizes to the nucleus and binds DNA to modulate gene expression as a transcriptional regulator. FoxP3 downregulates IL-2 and IL-4 gene transcription and upregulates the expression of CD25 and CTLA-4.

Several genes regulated by FoxP3 are also target genes for transcription factor NFAT. NFAT upregulates IL-2, IL-4, CD25 and CTLA-4. The interaction of FoxP3 and NFAT was supported by the identification of forkhead binding domains adjacent to NFAT transcription factor binding sites in the promoters of several cytokine genes (including IL-2, IL-4 and TNF). FoxP3 protein can form a cooperative complex with NFAT on DNA, acting as a transcription factor to both repress genes involved in T-cell activation, such as IL-2 and IL-4 and activate those required for the T-reg genetic programming, such as CD25 and CTLA-4.

Structure-based mutations of FoxP3, disrupting its interaction with NFAT, were shown to decrease its ability to repress IL-2. Mutations in this interface also interfere with the ability of retrovirally transduced FoxP3 to upregulate CTLA-4 and to a lesser extent CD25, CD103 and GITR expression. These mutations go on to impair the regulatory function of FoxP3 expressing Tregs, which become incapable of preventing autoimmunity. Thus, the transcriptional role of FoxP3 in developing suppressor function depends crucially on its integrity (48).

Mutations

Mutations in FoxP3 cause both the human X-linked fatal autoimmune disease “immune dysregulation, polyendocrinopathy, enterophathy, X-linked” (IPEX) syndrome (49) and an analogous X-linked pathology in the scurfy (sf) mutant mouse (50). Both human IPEX and the sf mouse are characterized by deficiency of Treg cell

function, due to FoxP3 malfunction, highlighting the importance of FoxP3 in Treg cell development (51).

Targets of FoxP3

By using Chip, it was found that the promoters of 1,119 genes are direct targets of FoxP3 binding, including promoters for IL-2, CD25 and GITR. Furthermore, FoxP3 targets seem to be associated with TCR signaling and activation of T effector cells. In order to determine whether FoxP3 binding truly affects the expression of these genes, Marson et al. performed DNA microarray profiling. They found that in unstimulated cells, there were only a few differences in gene expression either in the presence or absence of FoxP3 (125 differentially expressed genes in FoxP3+ versus FoxP3 hybridomas). However, in stimulated cells FoxP3 binding was associated with downregulation of target genes that are normally upregulated during TCR stimulation and T-cell activation. In conclusion, it was found that the targets of FoxP3 binding are mainly genes involved in TCR activation and binding of FoxP3 to its target genes appears to downregulate their expression (49).

Mechanisms of suppression

In vitro studies have shown that natural Tregs suppress the activation and/or proliferation and cytokine formation of CD4+ and CD8+ T-cells even in the absence of antigen presenting cells (APCs). Natural Tregs also suppress B-cell proliferation (52), immunoglobulin production (53) and class switch (52). Furthermore, they inhibit the cytotoxic functions of NK and NKT cells (53,54) and the function and maturation of DCs. Moreover, natural Tregs inhibit not only the activation and/or expansion of naïve T-cells but also the function of effector T-cells. They can also suppress memory T-cells although they more effectively control activation and/or expansion of naïve T-cells (55).

The induction of T-reg suppressive activity requires antigenic stimulation (i.e. activation signal through TCRs), whereas suppression that is exerted by activated Tregs is antigen non-specific (54,56). Thus, activated Tregs can inhibit a wide range of immune responses through bystander suppression. Several molecular events are important to understand how Tregs exert suppression on such various types of target cells and immune responses.

The suppression of the immune response by Tregs is induced through several mechanisms, as follows:

- ✓ Cell-to-cell contact:

As shown by in vitro experiments suppression is cell-to-cell contact-dependent. More precisely, it was found that Treg suppression is abolished when Tregs and T effector cells are separated by a semi-permeable membrane (54,56). T-cell accessory molecules, such as CTAL-4 and lymphocyte-activation gene 3 (LAG3), expressed by

Tregs, and CD80 and CD86 costimulatory molecules expressed by APCs contribute to this contact-dependent suppressive mechanism. There is evidence that CTLA-4 serves a key role in T-reg-mediated suppression in vivo and in vitro through its pathway. Direct suppression occurs through CTLA-4-dependent induction of the enzyme 2,3-dioxygenase (IDO) in DCs by interacting with their CD80 and CD86 molecules (57). IDO catalyses the conversion of tryptophan into kynurenine and other metabolites, which have potent immunosuppressive effects in the local environment of CDs by means of cytotoxicity or possibly by inducting de novo generation of Tregs from naive CD25-CD4+ T cells.

✓ Cytokines:

- IL-10: Many *in vivo* and *in vitro* studies indicate that IL-10 is needed for mediating suppression. Several *in vivo* experiments support the indispensable role of IL-10 in Treg suppression. In the study conducted by Kingsley et al. adoptive transfer of $CD4^+CD25^+$ T-cells into mice with allogenic skin grafts induced graft tolerance, but when injecting IL-10-receptor blocking antibody transplants are rapidly rejected (58).
- The data however, seem to be controversial as it was shown that human Tregs do not secrete IL-10 when cultured with phytohemagglutinin (PHA), IL-2 and feeder cells, but secreted TGF-β (Figure 6).
- TGF-β: As TGF-β is found on the membranes of murine and human Tregs (59), it is possible that this factor is part of cell-contact-dependent suppression. 80% of murine $CD4^+CD25^+$ T-cells express TGF-β on their surface when activated with high-dose anti-CD3 antibodies, irradiated T-cell-depleted APCs and IL-2. Treg membrane TGF-β is also involved in NK-cell suppression (40,60).
- IL-2: IL-2 receptor is constitutively expressed in most $FoxP3^+$ Tregs and in IL-2-deficient mice $FoxP3^+$ T-cells are substantially reduced. [60] Furthermore, anti-IL-2 antibody treatment specifically reduced the number of $CD25^+CD4^+$ T-cells, producing organ-specific autoimmune disease as observed after Treg depletion. These findings indicate that natural Tregs requires IL-2 for their maintenance and peripheral survival. The main source of IL-2 for Tregs seems to be the other T-cells (31).

✓ Cytotoxicity:

Another mechanism of suppression of equal importance is cytotoxicity. This is accomplished through perforin and granzyme A that kills T-cells, monocytes and DCs and through granzyme B that induces death to T-cells and B-cells (61).

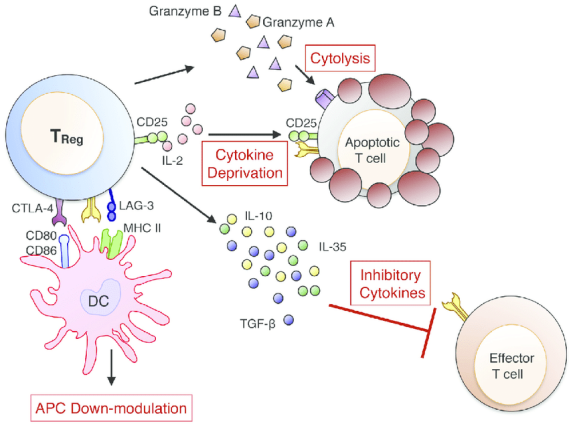


Figure 6: Mechanisms of suppression by Tregs

Hypothesis – Aim of the study

From previous studies of our laboratory we already know that 1) CIN patients display lower proportion of MDSCs in the PB and BM compared to healthy controls, that 2) MDSCs in CIN display normal T-cell suppression capacity, and that 3) CIN patients display higher PMN-MDSC accumulation in BM (vs PB) compared to healthy individuals. The low proportions of MDSC subsets may have a role in the persistence of the inflammatory processes associated with CIN. The accumulation of PMN-MDSCs in the BM may represent a compensatory mechanism to suppress the inflammatory processes within patients' BM (62).

As MDSCs induce the population of Tregs and these populations (i.e. MDSCs and Tregs) show a correlation in numbers in many conditions, we hypothesize that Tregs may also be altered in number and functionality in patients with CIN, and thus contribute in the sustained inflammatory process in the BM. Moreover, MDSCs may have impaired or altered property of inducing the Treg population in patients with CIN.

Materials and methods

1) Patients and Controls

40 patients from the outpatient Clinic of the Hematology Department of University Hospital of Heraklion diagnosed with CIN were enrolled in the study and their PB was studied. In 6 of these patients their BM was also studied. According to the criteria, CIN patients had absolute neutrophil counts (ANC) below 1800 x 106/L for a period of at least 3 months, had no clinical, serological or ultrasonic evidence of any underlying disease associated with neutropenia, no history of exposure to irradiation, use of chemical compounds or intake of drugs to which neutropenia might be ascribed, normal BM karyotype and negative antineutrophil antibodies. Cyclic cases were excluded by serial neutrophil enumerations. The control group consisted of 30 volunteers hematologically healthy and age- and sex-matched with the patients. All

patients had a routinely whole blood test and differential measurements. The proportion of PB and BM MDSCs and Tregs were evaluated by flow cytometry and analysis was conducted with the Kaluza software. Statistical analysis was done with the Graph Pad software and the Mann-Whitney test was used for unpaired data, the Wilcoxon test for paired data and the Spearman test for correlations. Our study protocols follow in detail.

CIN patients	Control group
PBMCs:40	PBMCs:30
BMMCs:6	

In order to estimate the number of both MDSCs and Tregs in the different groups of our study (CIN patients and healthy individuals) we proceeded with the immunophenotyping of PBMCs or BMMCs as follows:

2) Peripheral Blood Mononuclear Cells (PBMC) and Bone Marrow Mononuclear Cells (BMMCs) isolation

- BM aspirates/PB samples were collected in tubes containing EDTA.
- The samples were diluted 1:1 with plain RPMI 1640 (1X) Medium Gibco™ (THERMO-FISHER SCIENTIFIC).
- 6-8 mL of diluted sample was layered over 3-4 ml of Ficoll (Lymphoprep™-STEMCELL TECHNOLOGIES) in a 15 ml falcon tube.
- The tubes were centrifuged at RT, 20 min, 1500 rpm, brakes off.
- The mononuclear cell layer was transferred to a clean 15 ml tube.
- PBS (Phosphate-Buffered Saline) was added up to 15 ml.
- The tubes were centrifuged at 4C˚, 5 min, 1500 rpm, brakes on.
- PBS was added up to 3-5 ml.
- Cells were counted by using Trypan Blue and a hemocytometer.

3) Immunophenotyping - Cell staining

<u>Cell staining for MDSCs:</u>

- 10^6 cells were placed in flow cytometry (FC) 5ml tubes.
- 40 µl Fc blocking reagent [γ-Globulins, Human: From Cohn Fraction II, III Approx. 99% (electrophoresis) G4386-1G/SIGMA] were added.
- The samples were incubated for 10 min at 4°C in the dark.
- Monoclonal antibodies were added (10µl for PE and FITC – 5µl for PC5, PC7, ECD), vortex.
- Samples were incubated for 20 min at 4°C in the dark.

- Cells were washed with PBS and centrifuged at 400g for 5 minutes at room temperature. The supernatant was discarded.
- Stained cells were resuspended in the appropriate volume of Flow Cytometry Staining Buffer [300 µl PFA (Paraformaldehyde) 1%].
- The samples were analyzed in flow cytometer (Beckman Coulter Cytomix FC500).

Panel:

- CD33-PC7/CD15-PC5/DR-ECD/CD14-PE/CD11b-FITC

Marker	Fluorochrom	Clone
CD33	PC7	D3HL60.251
CD15	PC5	80H5
HLA DR	ECD	Immun-357
CD14	PE	RM052
CD11b	FITC	Bear1

Table 1: Fluorochrom bound Antibodies for MDSCs staining

Cell staining for Tregs

- 10^6 cells were placed in flow cytometry (FC) 5ml tubes.
- 40 µl Fc blocking reagent [γ-Globulins, Human: From Cohn Fraction II, III Approx. 99% (electrophoresis) G4386-1G/SIGMA] were added.
- The samples were incubated for 10 min at 4°C in the dark.
- Monoclonal antibodies and isotype controls were added (10µl for PE and FITC – 5µl for PC5, PC7, ECD), vortex.
- Samples were incubated for 20 min at 4°C in the dark.
- Cells were washed with PBS and centrifuged at 400g for 5 minutes at room temperature. The supernatant was discarded.
- 1 mL of Foxp3 Fixation/Permeabilization working solution (Invitrogen™
- eBioscience™ Foxp3 / Transcription Factor Staining Buffer, Set Catalog number: 00-5523-00) was added to each tube and pulse vortexed.
- The samples were incubated for 20 minutes at 4°C in the dark.
- 2 mL of 1X Permeabilization Buffer (Invitrogen™eBioscience™ Foxp3 / Transcription Factor Staining Buffer, Set Catalog number: 00-5523-00) were added to each tube and the samples were centrifuged at 400g for 5 minutes at room temperature. The supernatant was discarded.
- The pellet was resuspended in residual volume of 1X Permeabilization Buffer. This is typically 100 µL after decanting.
- Without washing, 5µl of the directly conjugated antibody for detection of intracellular antigen (FoxP3-FITC and isotype control) to cells and incubate for 30 minutes at 4°C in the dark.

- 2 mL of 1X Permeabilization Buffer were added to each tube and the samples centrifuged at 400g for 5 minutes at room temperature. The supernatant was discarded.
- Stained cells were resuspended in the appropriate volume of Flow Cytometry Staining Buffer [300 µl PFA (Paraformaldehyde) 1%].
- The samples were analyzed in flow cytometer (Beckman Coulter Cytomix FC500).

Panels:

- CD4-PC5/CD25-PE/FoxP3-FITC
- CD4-PC5/Control-PE/Control-FITC

Marker	Fluorochrom	Clone
CD4	PC5	13B8.2
CD25	PE	B1.49.9
FoxP3	FITC	236A/E7

Table 2: Fluorochrom bound Antibodies for Tregs staining

4) Flow cytometry principals

The flow cytometer is an instrument measuring multiple physical characteristics of a single cell such as size and granularity simultaneously as the cell flows in suspension through a measuring device. Flow cytometry depends on the light scattering features of the cells under investigation, which may be derived from dyes or fluorochrom bound monoclonal antibodies targeting either extracellular molecules located on the surface or intracellular molecules inside the cell (63).

According to Thermo Fisher Scientific, when a sample containing fluorescently labeled cells is uptaken by the instrument, it is mixed with a physiological buffer called sheath fluid and it is transferred to the flow chamber, where cells within the sample line up to a single-file stream, which is a critical step for single-cell analysis. One by one, cells pass through a narrow channel called the interrogation point where they meet the laser of the cytometer. When the laser light beam illuminates a single cell, some of the light will strike physical structures within the cell, causing the light to scatter. The forward scatter (FSC) provides information about the size of the cell, whereas the side scatter (SSC) gives information about the complexity of the cell (granules). The light from the laser that hits the cells nearly simultaneously excites all fluorophores linked with antibodies, previously attached on the surface or the interior receptors of cells, producing a fluorescence emission. All of this light is collected by the different light detectors depending on the wave lengths of the scattered light and is processed by the electronic component of the flow cytometer. After passing through the interrogation point, the cells are no longer of need and are placed into the waste

container with the assistance of the fluidics system. Data acquired by the sensors is collected and combined to build up a comprehensive picture of the sample. (Figure 7)

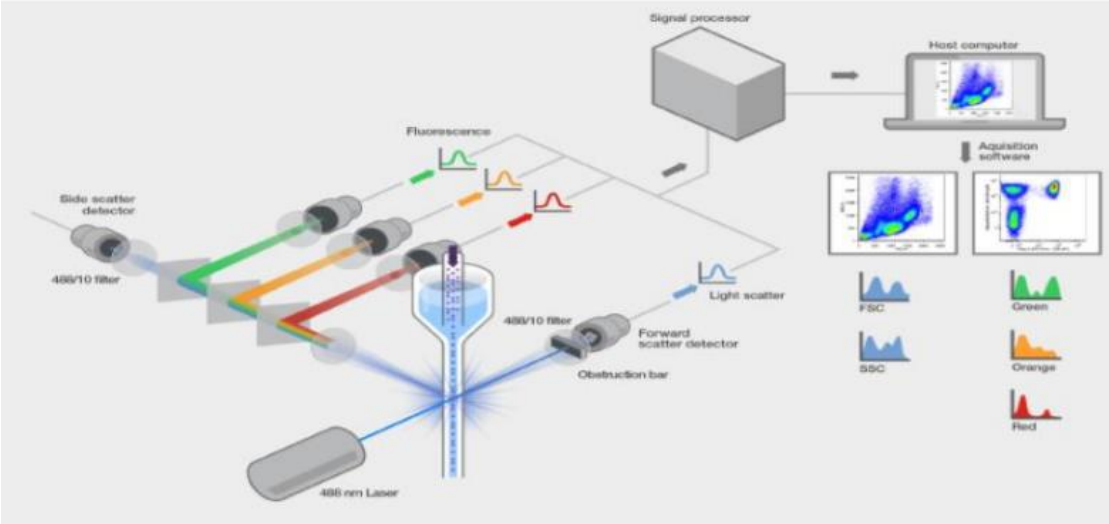


Figure 7: The working parts of the flow cytometer (image acquired from Thermo Fisher Scientific)

1) Flow Cytometry analysis strategy

After performing flow cytometry for all samples, the analysis of MDSCs (Figure 8) and Tregs (Figure 9) was performed with Kaluza Flow Cytometry Analysis Software and follows a back to back gating technique.

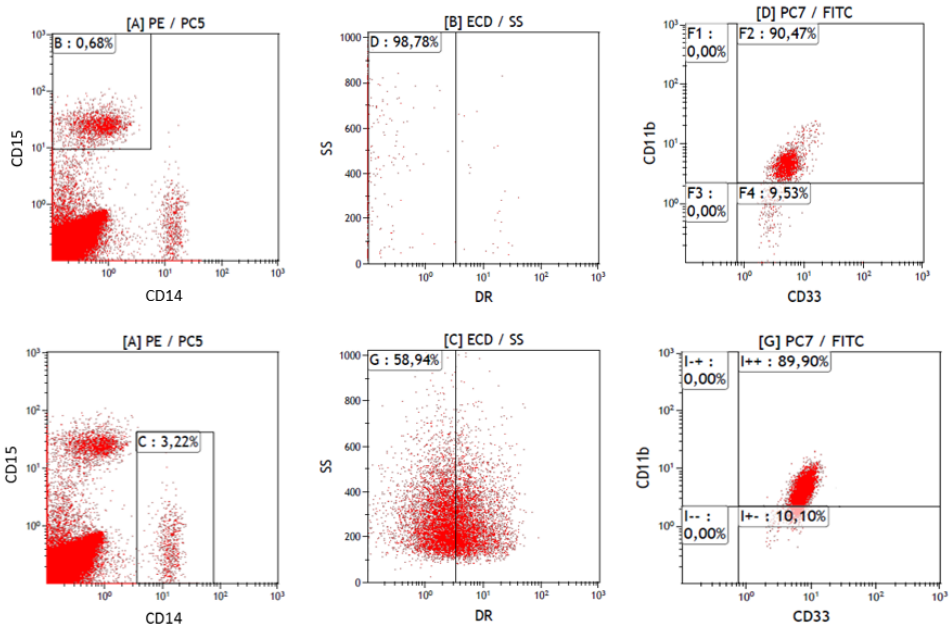


Figure 8: A representation of the FACS analysis for both MDSCs populations

For the PMN-MDSC population (upper graphs): From total PBMCs, the alive cells were gated. From the alive cells, the cells expressing the CD15 marker were gated (B). From the (B) population, the DR$^{-/low}$ cells were gated (D). From the (D) population, the cells expressing both the CD11b and CD33 markers were gated. This final population is the PMN-MDSCs population existing in the total PBMCs sample. For the M-MDSC population (lower graphs): From total PBMCs, the alive cells were gated. From the alive cells, the cells expressing the CD14 marker were gated. From the (C) population, the DR$^{-/low}$ cells were gated (G). From the (G) population, the cells expressing both the CD11b and CD33 were gated. This final population is the M-MDSCs population existing in the total PBMCs sample.

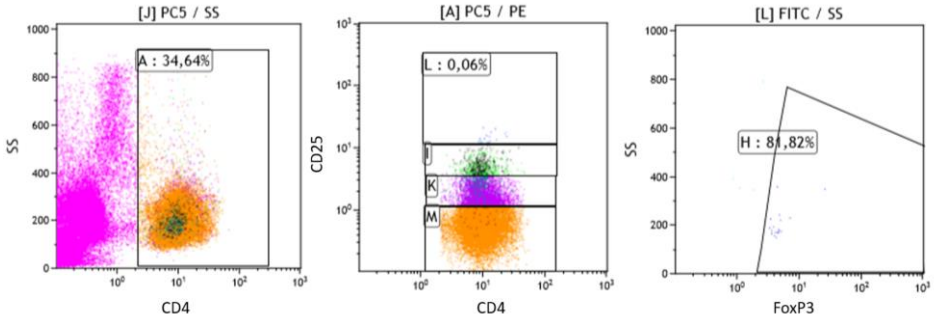


Figure 9: A representation of the FACS analysis for Tregs

From total PBMCs, the alive cells were gated. From the alive cells, the cells expressing the CD4 marker were gated (A). From the (A) population, the cells expressing very highly CD25 (CD25$^{high-high}$) (L), the cells expressing highly CD25 (CD25^{high}) (I), the cells expressing dimly CD25 (CD25^{dim}) (K) and the CD25 negative cells (M) were gated. In each subpopulation, the FoxP3^{+} cells were gated. Theoretically, Tregs are the FoxP3^{+} cells that exist in the populations (L) and (I).

5) Functional studies: T-Cell suppression assay

In order to estimate the suppressive activity of MDSCs in the two different study groups (CIN patients and control group) we proceeded with a T-cell suppression assay.

The suppression of T-cell proliferation by MDSCs was estimated by comparing the proliferation by means of CFSE (Carboxy-Fluorescein Succinimidyl Ester) staining of the anti-CD3/anti-CD28 activated T-cells cells in PBMCs and in PBMCs depleted from CD33+ cells, after 3 days of culture. The PBMCS depleted from CD33+ cells are expected to be devoid of MDSCs (Figure 10).

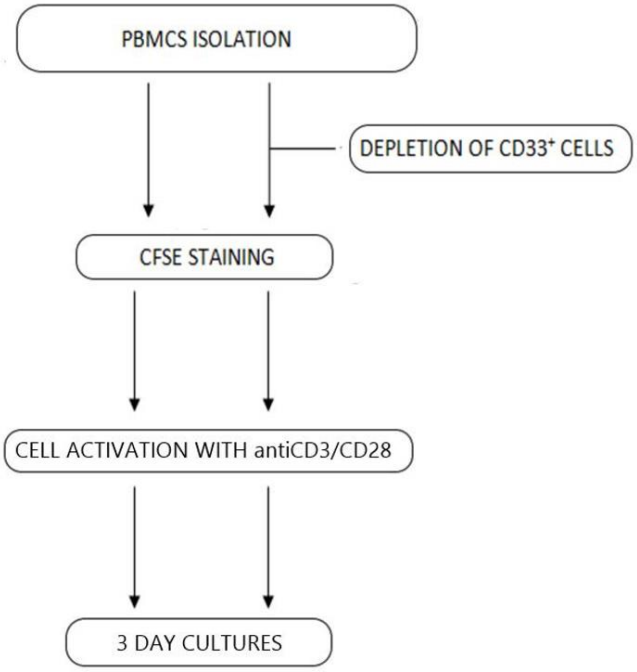


Figure 10: Flow chart of the process followed for the qualitative experiment

To monitor lymphocyte proliferation, with minimal disruption to cell viability and function we use an intracellular fluorescent dye, CFSE, which has the ability to stably label molecules within cells with each cell division resulting in a sequential halving of fluorescence (Figure 10). Initially, carboxy-fluorescein diacetate succinimidyl ester is non-fluorescent, owing to the presence of two acetate groups, but these groupings result in the compound being highly membrane permeant and thereby enables the dye to rapidly flux across the plasma membrane of cells. Once inside a cell, however, the acetate groups are rapidly removed by intracellular esterases to yield the highly fluorescent CFSE that is trapped inside cells owing to its reduced membrane permeability. Some of the CFSE conjugates are highly stable and remain inside cells. It is these fluorescent conjugates that persist within cells that are diluted between daughter cells following cell division, which allows lymphocyte proliferation to be monitored by flow cytometry. Usually, lymphocyte proliferation can be monitored by flow cytometry for up to eight divisions before CFSE fluorescence is decreased to the background fluorescence of unlabeled cells (64).

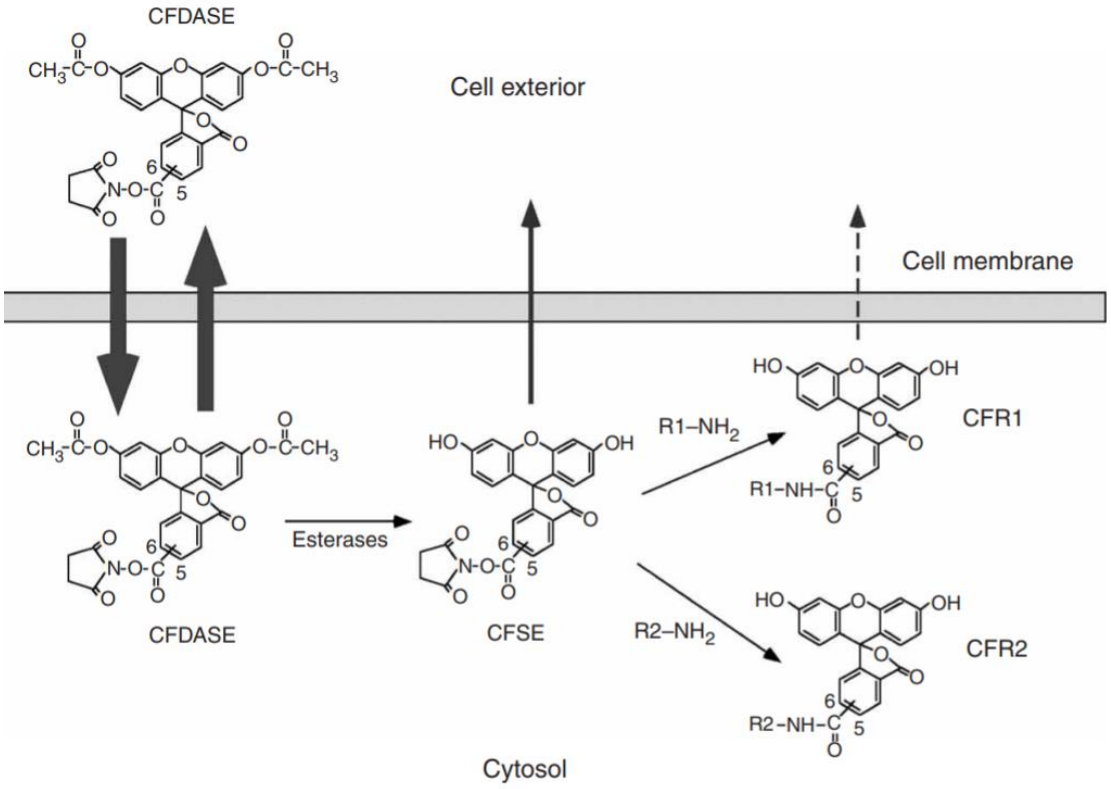


Figure 10: A schematic representation of the various molecular events that occur during the labeling of cells with CFDASE.

"CFR1, carboxyfluorescein conjugated molecule that is lost from the cell; CFR2, carboxyfluorescein conjugated molecule that is retained by the cell." (Quah, Warren, and Parish 2007)

PBMCs isolation

• PB samples were collected in EDTA containing tubes.

• Samples were diluted 1:1 with plain RPMI 1640 Medium Gibco™ (THERMO-FISHER SCIENTIFIC).

• 7-8 ml of diluted sample were layered over 4 ml of Ficoll (centrifuge gradient) (Lymphoprep™- STEMCELL TECHNOLOGIES) in a 15 ml falcon tube.

• Cells were centrifuged at RT for 30min, at 1800 rpm with no brakes.

• The mononuclear cell layer was transferred to a clean 50ml falcon tube.

• Cold MINIMACS buffer was added up to 30 ml [500ml of MINIMACS buffer contains 50ml ACD, 25ml BSA (Bovine Albumin Fraction V Solution 7.5% Gibco™ - Life Technologies), 20ml NaHCO3 (Sodium Bicarbonate Solution 7.5% Gibco™ - Life Technologies) and 405ml PBS]

• Cells were centrifuged at RT for 5 min at 1600 rpm with brakes on and the supernatant was discarded.

• The cell pellet was resuspended with the appropriate volume of MINIMACS buffer (10-20 mL) depending on the density of cells in each sample.

• The cells were counted by using Trypan Blue and a hemocytometer.

• Up to 4*106 PBMCs were stored in RPMI/10%FBS in 4°C (in 200μl → 250000 cells), for later use (part a).

• Up to 4*106 PBMCs were stored in 1ml PBS/5%FBS in 4°C, for later use (part b).

• Up to 20-30*106 PBMCs were stored in MINIMACS buffer for later use (part c).

CD33 depletion of PBMCs by immunomagnetic sorting

• 20-30*106 PBMCs in MINIMACS buffer (part c) were centrifuged at RT, for 5 min, at 1600 rpm with brakes on and the supernatant was discarded.

• Cells were incubated with 40-60μl (depending on the number of PBMCs) of anti-CD33 antibody conjugated with magnetic microbeads (MACS Miltenyi Biotec-clone AC104.3E3) for 15 min at 4°C according to the manufacturer's instructions.

• Cells were washed with MINIMACS buffer and Centrifuged at RT, for 5 min, at 1600 rpm, with brakes on in order to stop the labeling.

• Cells were resuspended in 1ml of MINIMACS buffer and passed through an LD column, previously washed with 2ml of MINIMACS buffer.

• The column was washed once with 1ml of MINIMACS buffer.

• The CD33- cells that flew through the LD column were gathered in a clean tube, resuspended and counted by using Trypan Blue and a hemocytometer.

• Up to 4*106 CD33- cells were stored in RPMI/10%FBS (in 200μl → 250000 cells), in 4°C for later use (part d).

• Up to 4*106 CD33- cells were stored in 1ml PBS/5%FBS in 4°C for later use (part e).

CFSE staining procedure

• Up to 4*106 PBMCs (part a) and 4*106 CD33- cells (part d) were diluted in 1ml PBS/5%FBS each.

• Cells were incubated for 10 min, with 100μl of CFSE (2 μM) (Cell-Trace CFSE Proliferation Kit-Thermo Fisher Scientific) in 37°C.

• 8-12 ml of PBS/5% FBS were added to stop the reaction.

• Cells were centrifuged at RT, 5 min, 1600 rpm, with brakes on and the supernatant was discarded.

• Cells were resuspended in PBS/5% FBS and counted by using Trypan Blue and a hemocytometer.

• 8-12ml of PBS/5% FBS were added and cells were washed by centrifugation at RT for 5 min, 1600 rpm, with brakes on.

• CFSE-stained PBMCs and CD33- cells were resuspended in RPMI/10% FBS (in 200μl → 250000 cells).

Evaluation of autofluorescence, and CFSE staining with flow cytometry for both PBMCs and CD33- cells (day 0)

To evaluate the autofluorescence baseline of both CFSE-unstained PBMCs and CFSE-unstained CD33-depleted PBMCs as well as the success of CFSE staining of both PBMCs and CD33-depleted PBMCs, all cell subpopulations were stained with fluorescent monoclonal antibodies conjugated with fluorochromes other than FITC (BECKMAN COULTER) according to the manufacturer's instructions and the previously described method, with the following combinations: CD5-PC5 (BL1a clone) / CD7-PE (8H8.1 clone).

Cell activation

PBMCs and CD33- cells were cultured for 3 days in a 48-well plate with 17 μl of the activating factors anti-CD3/anti-CD28 (STEMCELL TECHNOLOGIES–ImmunoCultTM Human CD3/CD28 T-Cell Activator). 0.25*106 cells were placed per well for each condition, in a 0.5 ml final volume after dilution with RPMI/10% FBS.

	PBMCs (MDSCs INCLUDED) W/O ACTIVATION	PBMCs (W/O $CD33^+$ CELLS) W/O ACTIVATION
UNSTAINED CELLS	A	D
CFSE STAINED CELLS	B	E
	PBMCs (MDSCs INCLUDED) WITH ANTICD3/ANTICD28 ACTIVATION	PBMCs (W/O $CD33^+$ CELLS) WITH ANTICD3/ANTI28 ACTIVATION
CFSE STAINED CELLS	C	F

Table 3: Culture conditions

The difference in the proliferation between CFSE stained PBMCs and CD33 depleted PBMCs following anti-CD3/anti-CD28 activation was expected to be mostly due to MDSCs.

Evaluation by Flow Cytometry in day 3

After 3 days of culture, cells were stained with fluorochrome conjugated antibodies and evaluated by flow cytometry.

A & D:

Unstained PBMCs and $CD33^-$ cells, respectively, without activation were used to evaluate in day 3 whether culture conditions induce autofluorescence. Cells were stained with the following fluorescent antibodies (BECKMAN COULTER): CD5-PC5 (BL1a clone) / CD7-PE (8H8.1 clone).

B & E:

CFSE stained PBMCs and $CD33^-$ cells, respectively, without activation were used to evaluate in day 3 whether culture conditions induce proliferation regardless of activating factors. Cells were stained with fluorescent antibodies (BECKMAN COULTER): CD5-PC5 (BL1a clone) / CD7-PE (8H8.1 clone).

C & F:

CFSE stained PBMCs and CD33- cells, respectively, activated by anti-CD3/anti-CD28 were used to compare proliferation with the same CFSE stained cell population on day 0 and with each other in day 3. Cells were stained with fluorescent antibodies (BECKMAN COULTER): CD5-PC5 (BL1a clone) / CD7-PE (8H8.1 clone).

6) Proliferation analysis

The suppressive activity of MDSCs amongst the study groups was estimated by using FSC Express 6 Plus Research Edition proliferation analysis program. The proliferation diagrams were created by gating the population of T-cells out of both CFSE stained PBMCs and CD33- cells on day 0 establishing in this way the time zero resting population of cells, and by gating the T-cell population out of both CFSE stained PBMCs and CD33- cells on day 3 depicting in that way the percentage of the proliferating cells as well as the cells that stayed in the resting condition (no-division). We assumed as T-cell population the CD5+ or CD7+ cells. Both populations, i.e. CD5 and CD7 expressing cells, were proven to be the same.

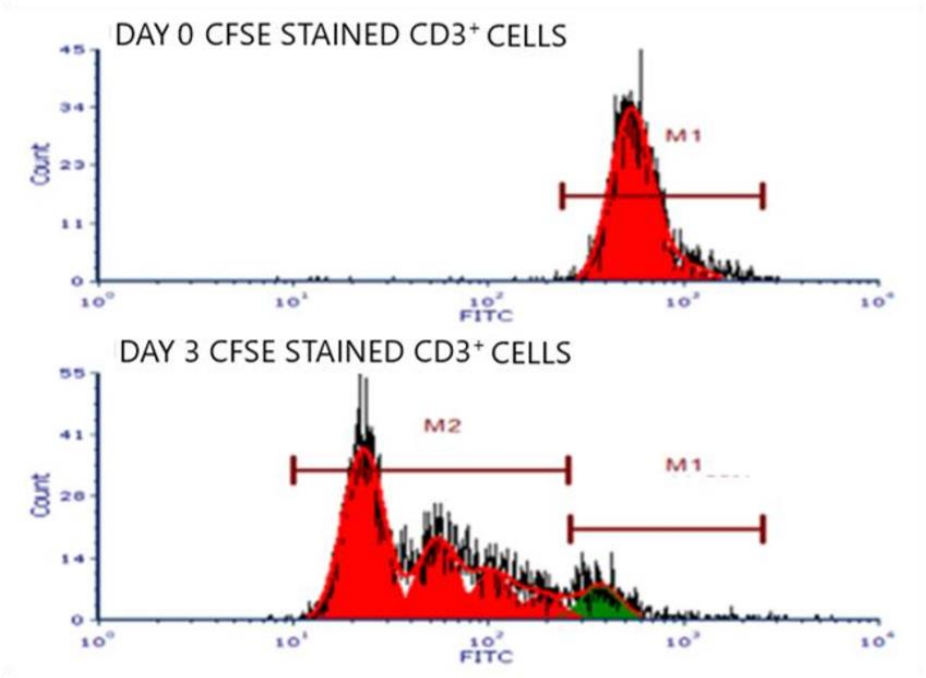


Figure 11: A representative image of the qualitative experiment analysis

7) Statistical analysis

Statistical analysis was performed with the Graph-Pad Prism 6 statistical program. Non parametric tests were performed, i.e. the Mann-Whitney test was used for unpaired data, the Wilcoxon test for paired data and the Spearman test for correlations.

Results

1) Cumulative data

The table below (Table 4) summarizes the results of the FCS analysis of the patient and normal samples.

	M-MDSCS %	PMN-MDSCS %	$CD25^{HIGH/HIGH}$ T-REGS %	$CD25^{HIGH}$ T-REGS %	$CD4^{+}CD25^{DIM}FOXP3^{+}$ %
NORMAL-PB	3,15021468	2,229750666	0,02	0,19	0,04
NORMAL-PB	0,951694427	1,476512583	0,05	0,22	0,07
NORMAL-PB	2,796223828	0,302969041	0	0	0,01
NORMAL-PB	3,61786902	6,488522852	0,03	0,11	0,01
NORMAL-PB	2,94923612	10,4123938	0,07	0,16	0,01
NORMAL-PB	0,579722944	0,11368539	0	0,01	0,01
NORMAL-PB	9,05054892	5,229046589	0,08	0,35	0,02
NORMAL-PB	5,718283628	2,492856853	0	0	0

NORMAL-PB	4,12325447	0,09605867	0,04	0,15	0,05
NORMAL-PB	6,434887346	1,536464311	0,05	0,29	0,06
NORMAL-PB	11,48412507	3,630459378	0,02	0,3	0,01
NORMAL-PB	3,24397878	13,82707003	0,09	0,09	0,03
NORMAL-PB	5,106184704	5,210721504	0,09	0,08	0,03
NORMAL-PB	5,957697661	4,617878482	0,07	0,53	0,05
NORMAL-PB	7,651783776	0,0702498	0,05	0,29	0,01
NORMAL-PB	6,892710972	0,11002368	0,03	0,18	0,02
NORMAL-PB	4,521067454	1,019048993	0,08	0,47	0,05
NORMAL-PB	2,448106895	8,56653504	0,06	0,17	0,04
NORMAL-PB	4,417127	0,770428333	0,1	0,39	0,04
NORMAL-PB	3,345080548	0,49192441	0,05	0,1	0,02
NORMAL-PB	5,122408896	2,236158137	0,11	0,36	0,06
NORMAL-PB	2,453200508	3,57967638	0,07	0,42	0,02
NORMAL-PB	1,847273003	2,067449832	0,06	0,04	0,03
NORMAL-PB	6,023095973	1,126353992	0,04	0,23	0,03
CIN-PB	3,552512418	2,40621216	0,02	0,01	0
CIN-PB	0,59187847	1,839777139	0	0	0
CIN-PB	4,773865694	3,226479656	0,03	0,29	0,14
CIN-PB	1,968279685	5,861738554	0,81	0,36	0,07
CIN-PB	2,008704442	3,94141528	0,05	0,31	0,02
CIN-PB	2,45179905	0,594905964	0,01	0,44	0,25
CIN-PB	0,894129671	0,539388324	0,09	0,47	0,02
CIN-PB	0,281771158	1,278659515	0,03	0,4	0,02
CIN-PB	1,227593131	2,2498504	0,55	0,45	0,01
CIN-PB	1,35468613	0,72871437	0,09	0,34	0,02
CIN-PB	2,82044539	0,903224193	0,17	0,23	0,01
CIN-PB	6,331849368	0,67535886	0,07	0,63	0,08
CIN-PB	4,312329021	0,491182623	0,05	0,05	0
CIN-PB	3,434342713	1,538769725	0,13	0,49	0,02
CIN-PB	2,284225709	0,355674825	0,01	0,52	0,29
CIN-PB	4,190641965	0,663956713	0,06	0,28	0,03
CIN-PB	4,027183815	0,026716736	0,04	0,47	0,04
CIN-PB	1,398318768	4,244530214	0,02	0,33	0,01
CIN-PB	0,663449631	0,070166574	0,23	0,23	0
CIN-PB	8,200688044	0,008332803	0,07	0,11	0,04
CIN-PB	4,500326002	0,3105552	0,14	0,7	0
CIN-PB	1,419066277	1,746966211	0,36	0,68	0,02
CIN-PB	1,31278696	8,05987216	0,25	0,59	0,03
CIN-PB	2,284225709	0,032926463	0,01	0,52	0,29
CIN-PB	1,319626885	3,301344189	0,1	0,47	0,08
CIN-PB	1,86662328	1,055059451	0,12	0,35	0,09
CIN-PB	3,535561453	2,56118499	0,13	0,04	0,02
CIN-PB	11,98606033	7,207670574	0,07	0,33	0,09

CIN-PB	6,295718286	0,313777168	0,08	0,5	0,04
CIN-PB	5,632711243	1,98853152	0,1	0,09	0,03
CIN-PB	1,434331707	1,4155299	0,12	0,83	0,02
CIN-PB	7,090879641	2,413623643	0,09	0,51	0,03
CIN-PB	6,148755963	0,883022364	0,11	0,58	0,05
CIN-PB	2,578155026	1,62500771	0,04	0,21	0,01
CIN-PB	3,789635498	0,647633742	0,02	0,08	0,02
CIN-PB	10,58710853	0,654252242	0,11	0,35	0,08
CIN-PB	2,736384341	0,107041662	0	0,08	0,12
CIN-PB	0,339012994	1,487885108	0,1	0,18	0,04
CIN-BM	2,516077166	27,28702233	0,85	0,02	0,01
CIN-BM	1,278836253	29,19986215	0,04	0,26	0,02
CIN-BM	0,081824712	0,425170305	0,11	0,34	0,01
CIN-BM	18,68911809	0,053301316	0,11	0,39	0,01
CIN-BM	0,54088839	4,688103967	0,11	0,15	0,02

Table 4: FCS analysis results of the CIN patient and normal samples

2) M-MDSCs & PMN-MDSCs in PB & BM of CIN patients & healthy controls

In order to compare M-MDSCs in the PB of patients and controls we used the non-parametric Mann Whitney test. As already proven from previous studies, CIN patients displayed in this cohort again decreased proportion (Table 5 & Figure 12) of M-MDSCs in the PBMC fraction (3.425 ± 2.715) compared to the healthy controls (4.420 ± 2.455; p=0.041). As far as the BM M-MDSCs are concerned, paired analysis was carried out, i.e. Wilcoxon test. However, because of the small sample and the absence of normal BM controls, no statistically significant difference was found (Table 6 & Figure 13).

PB M-MDSCs	**CIN**	**Normal**
Number of values	39	27
Minimum	0,2818	0,5797
25% Percentile	1,398	2,796
Median	2,578	4,105
75% Percentile	4,500	5,958
Maximum	11,99	11,48
Mean	3,425	4,420
Std. Deviation	2,715	2,455
Std. Error of Mean	0,4347	0,4724
Lower 95% CI of mean	2,545	3,449
Upper 95% CI of mean	4,305	5,391
Sum	133,6	119,3

Table 5: PB M-MDSCs statistics

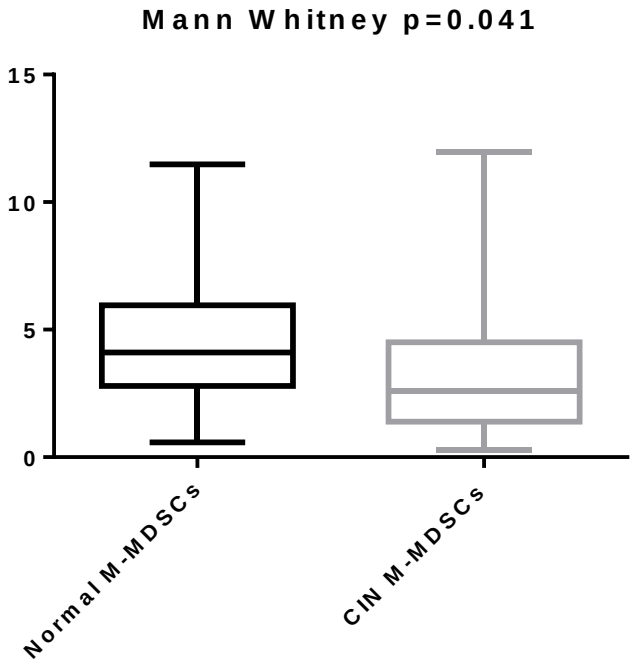


Figure 12: PB M-MDSCs in Normal vs CIN patients

M-MDSCs	**BM**	**PB**
Number of values	6	6
Minimum	0,08183	0,2818
25% Percentile	0,4261	0,3247
Median	1,363	1,957
75% Percentile	6,559	3,557
Maximum	18,69	8,201
Mean	4,092	2,457
Std. Deviation	7,199	2,929
Std. Error of Mean	2,939	1,196
Lower 95% CI of mean	-3,463	-0,6168
Upper 95% CI of mean	11,65	5,532
Sum	24,55	14,74

Table 6: PB and BM M-MDSCs statistics in CIN patients

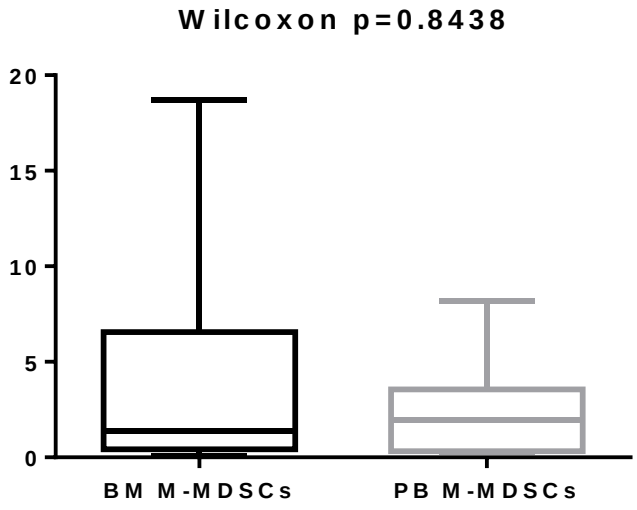


Figure 13: PB vs BM M-MDSCs in CIN patients

PMN-MDSCs in the PBMC fraction of the patients (Table 7 & Figure 14) were also decreased compared to healthy controls, but this difference was on the threshold of not statistically significant (p=0.0514). As far as the BM PMN-MDSCs are concerned, paired analysis was performed, i.e. Wilcoxon test. Despite the fact that in our previously described larger cohort PMN-MDSCs were proven to accumulate in the BM of CIN patients (62), because of the small sample and the absence of normal BM controls no statistically significant difference was found (Table 8 & Figure 15) in this small cohort of 6 patients. However, the trend is also obvious in this small cohort of patients, where the mean in the BM is 12,12% compared to 2,187% in the PB.

PB PMN-MDSCs	**CIN**	**Normal**
Number of values	39	27
Minimum	0,008333	0,07025
25% Percentile	0,5394	0,7704
Median	1,055	2,230
75% Percentile	2,406	5,211
Maximum	8,060	13,83
Mean	1,744	3,341
Std. Deviation	1,910	3,437
Std. Error of Mean	0,3058	0,6615
Lower 95% CI of mean	1,125	1,981
Upper 95% CI of mean	2,363	4,701
Sum	68,00	90,21

Table 7: PB PMN-MDSCs statistics

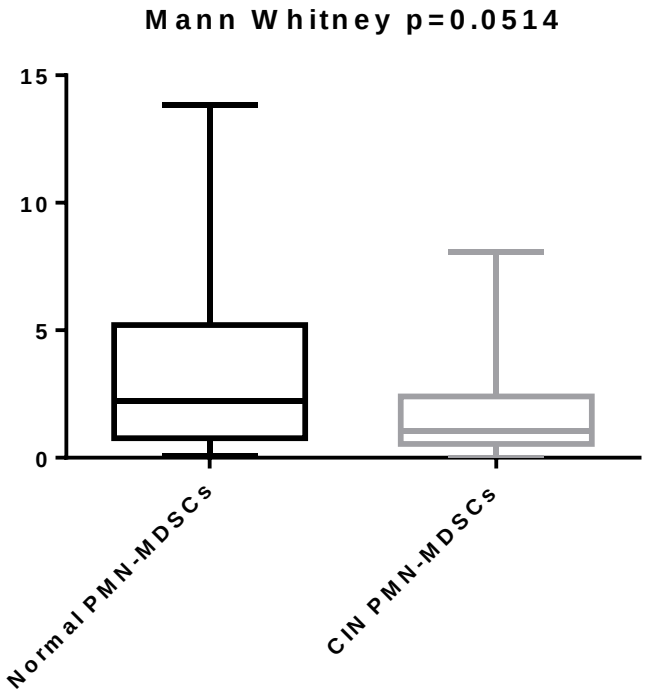


Figure 14: PB PMN-MDSCs in Normal vs CIN patients

PMN-MDSCs	**BM**	**PB**
Number of values	6	6
Minimum	0,05330	0,008333
25% Percentile	0,3322	0,4096
Median	7,890	1,383
75% Percentile	27,77	4,421
Maximum	29,20	5,862
Mean	12,12	2,187
Std. Deviation	13,12	2,251
Std. Error of Mean	5,355	0,9191
Lower 95% CI of mean	-1,642	-0,1758
Upper 95% CI of mean	25,89	4,550
Sum	72,75	13,12

Table 8: PB and BM PMN-MDSCs statistics in CIN patients

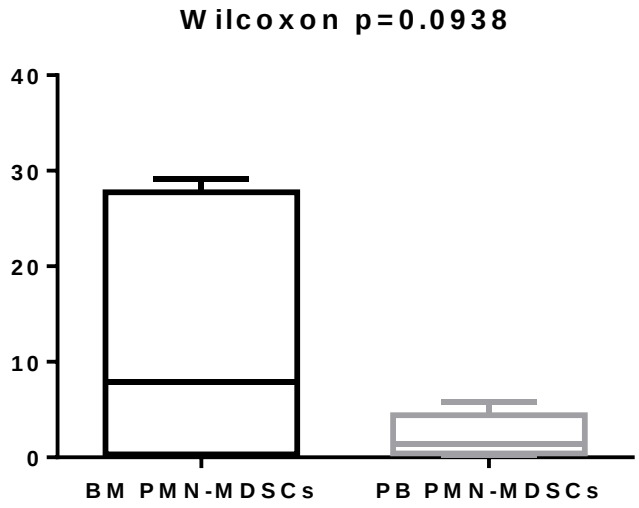


Figure 15: PB vs BM PMN-MDSCs in CIN patients

3) Tregs in PB & BM of CIN patients & healthy controls

In order to compare Tregs in the PB of patients and controls we used the non-parametric Mann Whitney test. CIN patients displayed increased proportion (Table 9 & Figure 16) of $CD4^{+}CD25^{high\text{-}high}FoxP3^{+}$ cells in the PBMC fraction (0.1163 ± 0.1531) compared to the healthy controls (0.05 ± 0.03088; p=0.0228). $CD4^{+}CD25^{high}FoxP3^{+}$ cells in the PBMC fraction (Table 10 & Figure 17) of the patients (0.3455 ± 0.2085) were also increased compared to healthy controls (0.1952 ± 0.1525; p=0.0032). We assumed that the population of Tregs is the sum of $CD4^{+}CD25^{high\text{-}high}FoxP3^{+}$ and $CD4^{+}CD25^{high}FoxP3^{+}$ cells and we found that Tregs are increased in the PBMC fraction (Table 11 & Figure 18) of the patients (0.4618 ± 0.2843) compared to the healthy controls (0.2452 ± 0.1720; p=0.0011). No statistically significant difference was found between the proportion of the $CD4^{+}CD25^{dim}FoxP3^{+}$ cells in the PBMC fraction of the patients and the healthy controls (Table 12 & Figure 19).

$CD25^{high\text{-}high}$Tregs	**Normal**	**CIN**
Number of values	27	40
Minimum	0,0	0,0
25% Percentile	0,0300	0,0300
Median	0,0500	0,0850
75% Percentile	0,0700	0,1275
Maximum	0,1100	0,8100
Mean	0,0500	0,1163
Std. Deviation	0,03088	0,1531
Std. Error of Mean	0,005944	0,02421
Lower 95% CI of mean	0,03778	0,06728
Upper 95% CI of mean	0,06222	0,1652
Sum	1,350	4,650

Table 9: PB $CD4^{+}CD25^{high\text{-}high}FoxP3^{+}$ cells statistics

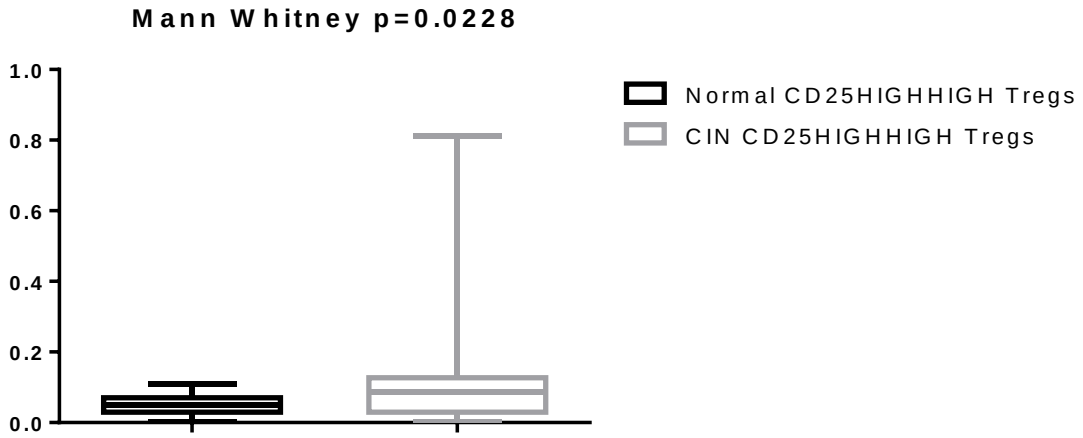


Figure 16: PB $CD4^{+}CD25^{high\text{-}high}FoxP3^{+}$ cells in Normal vs CIN patients

$CD25^{high}$Tregs	Normal	CIN
Number of values	27	40
Minimum	0,0	0,0
25% Percentile	0,0700	0,1800
Median	0,1700	0,3450
75% Percentile	0,3000	0,4975
Maximum	0,5300	0,8300
Mean	0,1952	0,3455
Std. Deviation	0,1525	0,2085
Std. Error of Mean	0,02935	0,03297
Lower 95% CI of mean	0,1348	0,2788
Upper 95% CI of mean	0,2555	0,4122
Sum	5,270	13,82

Table 10: PB $CD4^{+}CD25^{high}FoxP3^{+}$ cells statistics

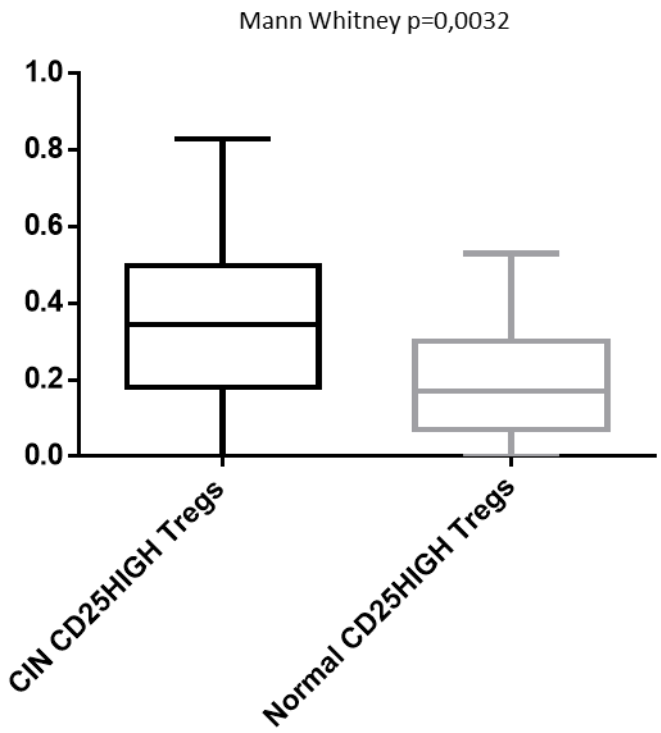


Figure 17: PB $CD4^{+}CD25^{high}FoxP3^{+}$ cells in Normal vs CIN patients

Tregs	**Normal**	**CIN**
Number of values	27	40
Minimum	0,0	0,0
25% Percentile	0,1000	0,2575
Median	0,2100	0,4400
75% Percentile	0,3400	0,5950
Maximum	0,6000	1,170
Mean	0,2452	0,4618
Std. Deviation	0,1720	0,2843
Std. Error of Mean	0,03310	0,04496
Lower 95% CI of mean	0,1771	0,3708
Upper 95% CI of mean	0,3132	0,5527
Sum	6,620	18,47

Table 11: PB Tregs statistics

Figure 18: PB CD4^{+}CD25^{high}FoxP3^{+} cells in Normal vs CIN patients

CD4+CD25dimFoxP3+ cells	Normal	CIN
Number of values	27	40
Minimum	0,0	0,0
25% Percentile	0,0100	0,0125
Median	0,0300	0,0300
75% Percentile	0,0400	0,0775
Maximum	0,0700	0,2900
Mean	0,02926	0,0545
Std. Deviation	0,01880	0,07253
Std. Error of Mean	0,003617	0,01147
Lower 95% CI of mean	0,02182	0,03130
Upper 95% CI of mean	0,03669	0,07770
Sum	0,7900	2,180

Table 12: PB CD4^{+}CD25^{dim}FoxP3^{+} cells statistics

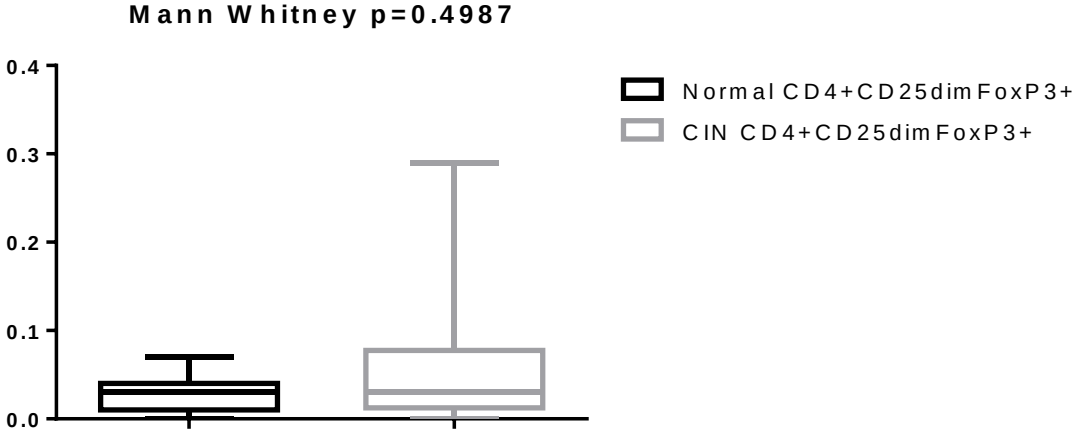


Figure 19: PB $CD4^{+}CD25^{dim}FoxP3^{+}$ cells in Normal vs CIN patients

Paired analysis showed that the proportion of the populations did not differ in the BMMC compared to PBMC fraction in both $CD4^{+}CD25^{high\text{-}high}FoxP3^{+}$ cells (Table 13 & Figure 20) of the patients and $CD4^{+}CD25^{high}FoxP3^{+}$ cells (Table 14 & Figure 21) of the patients. Similarly, the sum of the two populations, which we assumed to be the Tregs (Table 15 & Figure 22), did not differ between the BM and the PB of the patients. No statistically significant difference was also found for the $CD4^{+}CD25^{dim}FoxP3^{+}$ cells (Table 16 & Figure 23). However, these cells tend to be in very low numbers in the BM of our cohort.

$CD25^{high\text{-}high}$Tregs	**BM**	**PB**
Number of values	5	5
Minimum	0,0400	0,0300
25% Percentile	0,0750	0,0400
Median	0,1100	0,0700
75% Percentile	0,4800	0,4550
Maximum	0,8500	0,8100
Mean	0,2440	0,2120
Std. Deviation	0,3401	0,3353
Std. Error of Mean	0,1521	0,1499
Lower 95% CI of mean	-0,1783	-0,2043
Upper 95% CI of mean	0,6663	0,6283
Sum	1,220	1,060

Table 13: PB and BM $CD4^{+}CD25^{high\text{-}high}FoxP3^{+}$ cells statistics in CIN patients

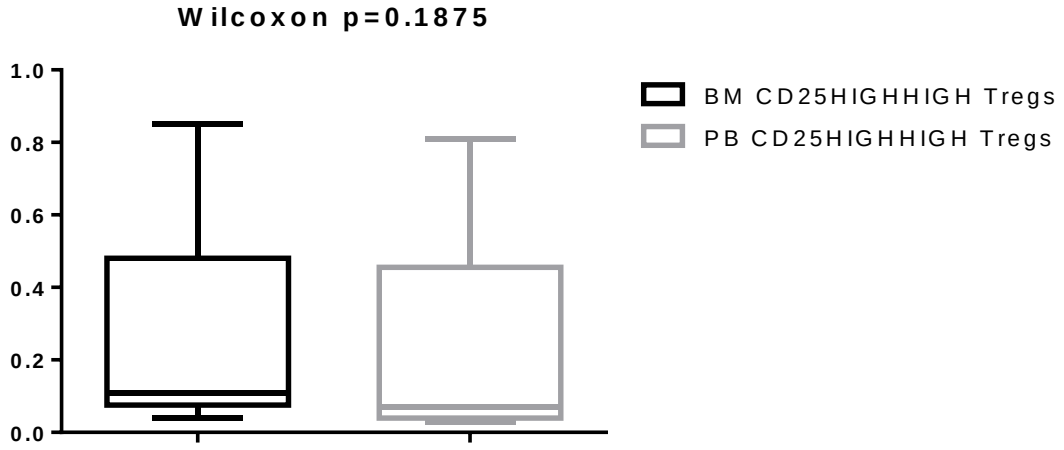


Figure 20: PB vs BM $CD4^{+}CD25^{high\text{-}high}FoxP3^{+}$ cells in CIN patients

$CD25^{high}$Tregs	**BM**	**PB**

Number of values	5	5
Minimum	0,0200	0,1100
25% Percentile	0,0850	0,1450
Median	0,2600	0,3100
75% Percentile	0,3650	0,3800
Maximum	0,3900	0,4000
Mean	0,2320	0,2720
Std. Deviation	0,1492	0,1228
Std. Error of Mean	0,06674	0,05490
Lower 95% CI of mean	0,04670	0,1196
Upper 95% CI of mean	0,4173	0,4244
Sum	1,160	1,360

Table 14: PB and BM CD4^{+}CD25^{high}FoxP3^{+} cells statistics in CIN patients

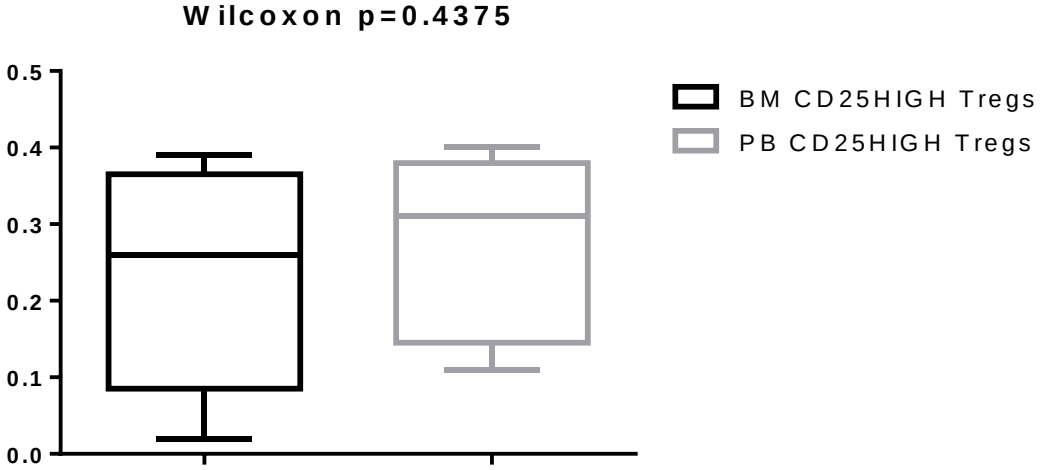


Figure 21: PB vs BM CD4^{+}CD25^{high}FoxP3^{+} cells in CIN patients

Tregs	**BM**	**PB**
Number of values	5	5
Minimum	0,2600	0,1800
25% Percentile	0,2800	0,2300
Median	0,4500	0,3600
75% Percentile	0,6850	0,8000
Maximum	0,8700	1,170
Mean	0,4760	0,4840
Std. Deviation	0,2419	0,3946
Std. Error of Mean	0,1082	0,1765
Lower 95% CI of mean	0,1756	-0,005996
Upper 95% CI of mean	0,7764	0,9740
Sum	2,380	2,420

Table 15: PB and BM Tregs statistics in CIN patients

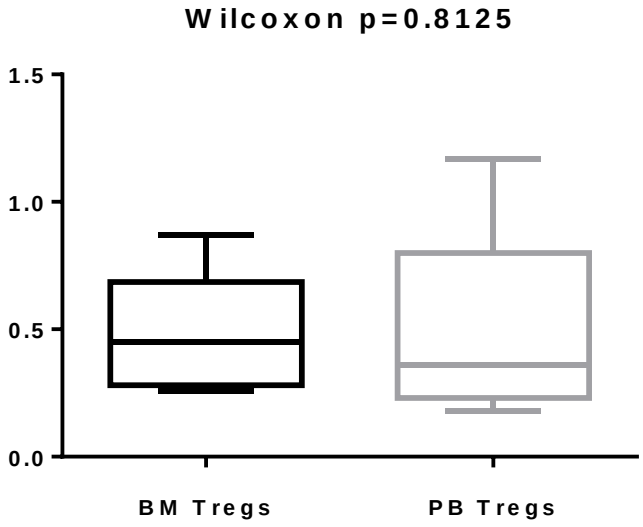


Figure 22: PB vs BM Tregs in CIN patients

$CD4^+CD25^{dim}FoxP3^+$ cells	**BM**	**PB**
Number of values	5	5
Minimum	0,0100	0,1800
25% Percentile	0,0100	0,2300
Median	0,0100	0,3600
75% Percentile	0,0200	0,8000
Maximum	0,0200	1,170
Mean	0,0140	0,4840
Std. Deviation	0,005477	0,3946
Std. Error of Mean	0,002449	0,1765
Lower 95% CI of mean	0,007199	-0,005996
Upper 95% CI of mean	0,02080	0,9740
Sum	0,0700	2,420

Table 16: PB and BM $CD4^+CD25^{dim}FoxP3^+$ cells statistics in CIN patients

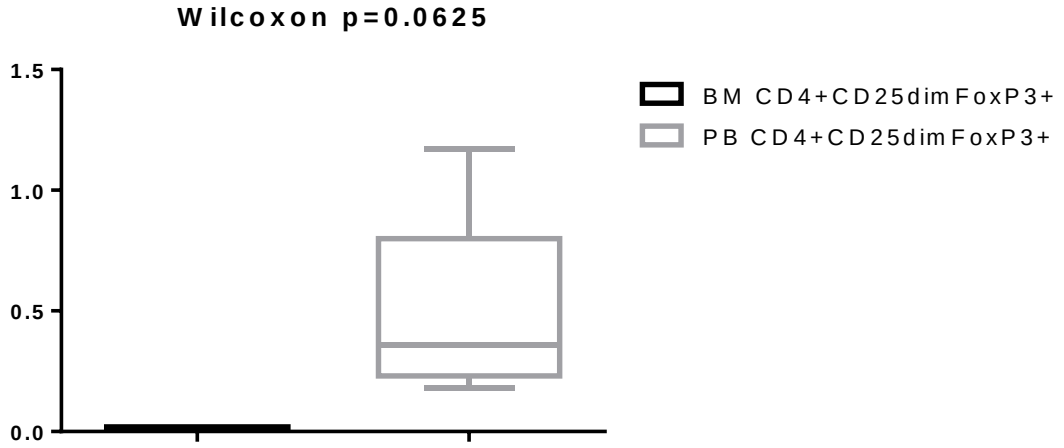


Figure 23: PB vs BM $CD4^{+}CD25^{dim}FoxP3^{+}$ cells in CIN patients

4) Tregs vs MDSCs in patients

In order to evaluate whether the populations of MDSCs correlated with the population of Tregs we conducted the non-parametric Spearman correlation test. No significant correlations were found between M-MDSCs and $CD25^{high\text{-}high}$ Tregs (Figure 24), $CD25^{high}$ Tregs (Figure 25), or the sum of Tregs (Figure 26). No significant correlations were found between PMN-MDSCs and $CD25^{high}$ Tregs (Figure 27), or the sum of Tregs (Figure 28). However, a positive correlation (Figure 29) was found between the proportion of PMN-MDSCs and the number of $CD25^{high\text{-}high}$ Tregs (r=0.3683, p=0.0229). This may suggest a role for PMN-MDSCs in the *in vivo* expansion of Tregs in our patients, as these cells may induce normally Treg proliferation.

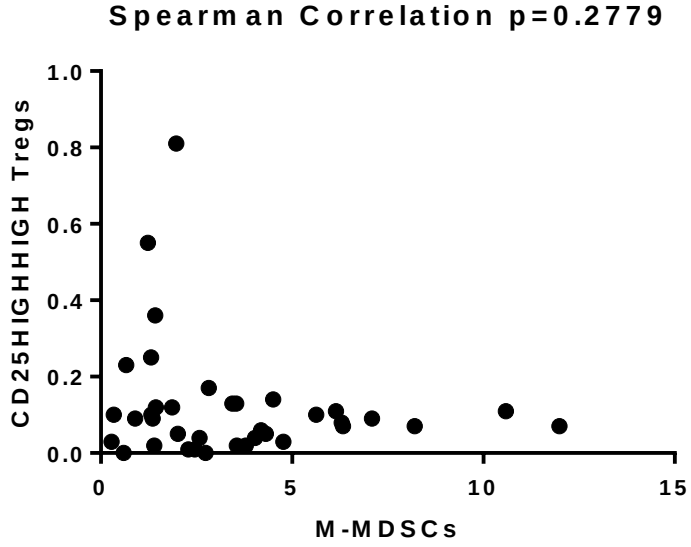


Figure 24: $CD4^{+}CD25^{high\text{-}high}FoxP3^{+}$ cells vs M-MDSCs in CIN patients

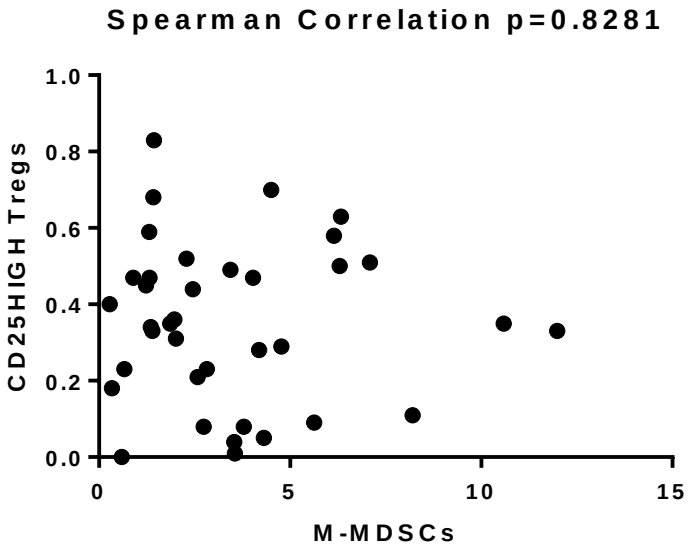


Figure 25: $CD4^{+}CD25^{high}FoxP3^{+}$ cells vs M-MDSCs in CIN patients

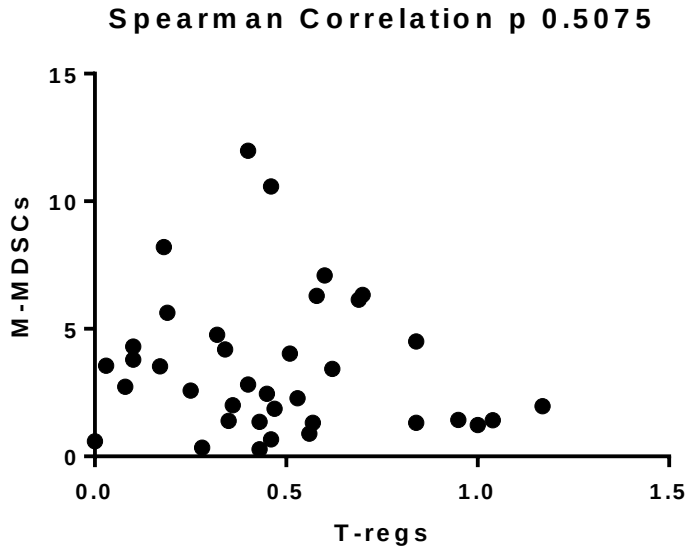


Figure 26: Tregs vs M-MDSCs in CIN patients

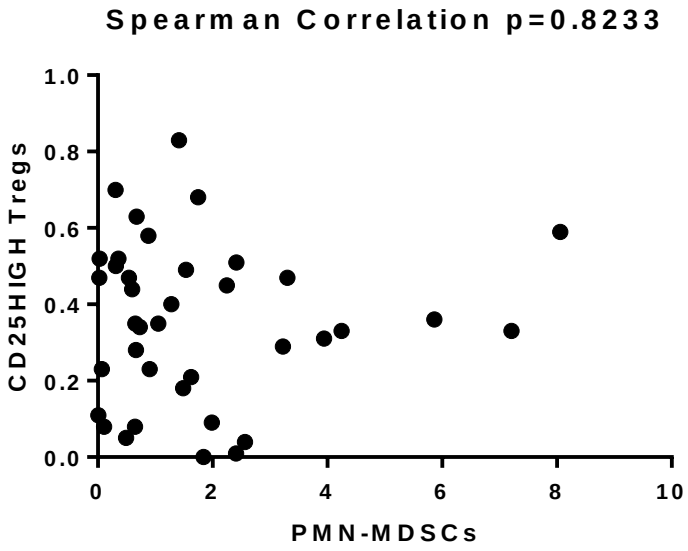


Figure 27: $CD4^{+}CD25^{high}FoxP3^{+}$ cells vs PMN-MDSCs in CIN patients

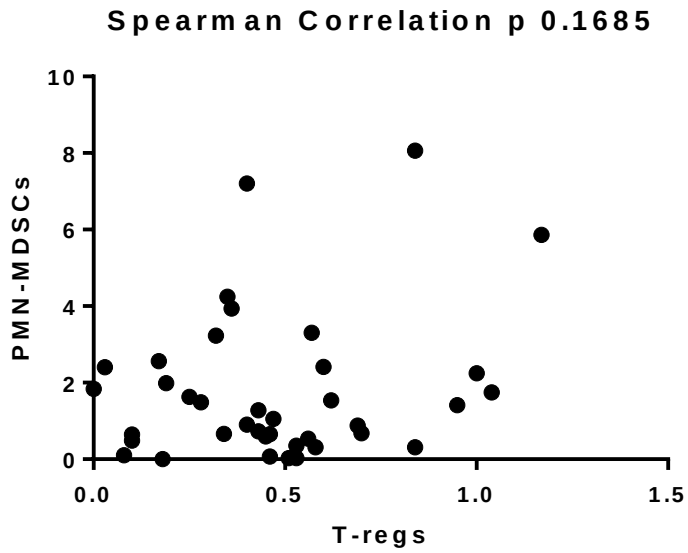


Figure 28: Tregs vs PMN-MDSCs in CIN patients

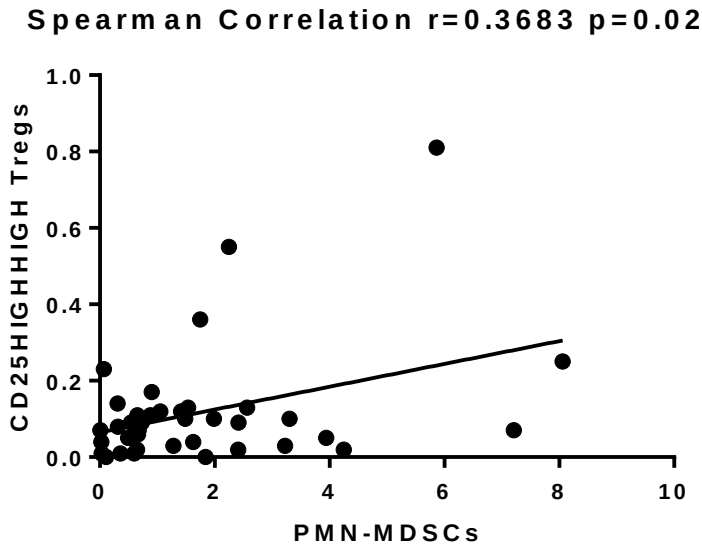
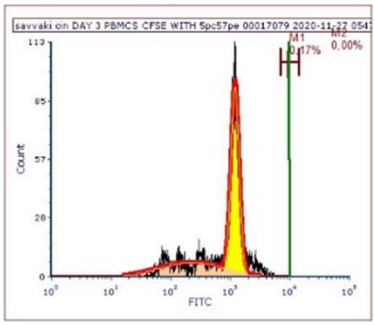


Figure 29: $CD4^{+}CD25^{high\text{-}high}FoxP3^{+}$ cells vs PMN-MDSCs in CIN patients

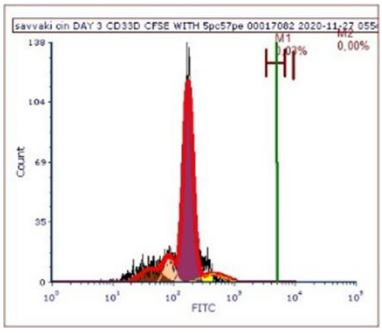
5) MDSCs capacity to suppress T-cell proliferation

Within the context of this master thesis we conducted functional studies as described above in the Materials & methods section for 5 CIN patients and confirmed, as previously described (62), that CIN-MDSCs display normal capacity to suppress T-cell proliferation in vitro. This was indicated by the T-cell generations in the presence or absence of patient MDSCs. PBMCs, where MDSCs are theoretically present, showed presence of undivided cells (5-90%), and decrease in generations (1-3 less generations) and dividing cells (5-90%), compared to CD33 depleted PBMCs, where MDSCs are theoretically absent. Figure 30 shows a characteristic picture of the T-cell suppression assay.



Generation	Peak Channel	Peak Value	Log Std Dev	Log CV	Peak Ratio	# of Cells
Undivided Cells	9478,60	0,00	0,00	0,00	0,00	0
Generation 1	4693,20	0,00	0,00	0,00	0,50	0
Generation 2	3262,47	0,00	0,00	0,00	0,70	0
Generation 3	1186,37	91,00	0,07	2,31	0,37	4098
Generation 4	860,10	0,00	0,00	0,00	0,72	0
Generation 5	441,62	0,00	0,00	0,00	0,51	0
Generation 6	148,55	7,00	0,57	26,34	0,51	2319

PBMCs day 3



Generation	Peak Channel	Peak Value	Log Std Dev	Log CV	Peak Ratio	# of Cells
Undivided Cells	4865,76	0,00	0,00	0,00	0,00	0
Generation 1	3184,57	0,00	0,00	0,00	0,65	0
Generation 2	892,79	0,00	0,00	0,00	0,28	0
Generation 3	395,96	5,00	0,21	8,08	0,49	553
Generation 4	382,43	0,00	0,00	0,00	0,87	0
Generation 5	170,01	115,00	0,08	3,45	0,45	5640
Generation 6	80,58	13,00	0,09	4,70	0,50	716
Generation 7	41,79	8,00	0,18	10,92	0,51	792

CD33 depleted cells day 3

Figure 30: T-cell proliferation after 3 days of culture of PBMCs and CD33 depleted cells of a CIN patient

Conclusions

The number of MDSCs in patients with CIN differs from that in healthy individuals, and contributes to the pathophysiology of the disease. As described before in patients with CIN, an inflammatory BM microenvironment is observed with the presence of activated oligoclonal T-lymphocytes and increased levels of inflammatory/apoptotic agents. Elevated levels of pro-inflammatory monocytes are also found in the PB of these patients, further contributing to prolonged inflammation. Also, CIN patients display higher PMN-MDSC accumulation in BM (vs PB) compared to healthy individuals. Moreover, CIN MDSCs display normal capacity to suppress T-cell proliferation *in vitro* (8,62).

Our hypothesis is that reduced rates of MDSCs contribute to the maintenance or even the initiation of this inflammatory BM microenvironment, due to the reduced immunosuppression they offer. If immune regulation depends on the balance of immunosuppressive and immunostimulatory signals, then a decrease in the MDSCs population may contribute to abnormal T-cell activation. Moreover, we assumed that Tregs may also be altered in number and functionality in patients with CIN, and thus contribute to the sustained inflammatory process in the BM. Finally, we hypothesized that MDSCs may have impaired/altered property of inducing the Treg population in patients with CIN.

Although the distinction between cause and effect is at this point impossible, the results of this study, and in particular M-MDSCs, seem to agree and reinforce the case. Specifically, the percentage of the population of M-MDSCs shows a significant decrease in the fraction of PBMCs in the peripheral blood of patients with CIN,

compared to healthy controls. PMN-MDSCs also show a decreasing trend, although no statistically significant difference was observed between CIN patients and controls. However, Tregs are higher in numbers in patients compared to normal controls. The number of $CD25^{high\text{-}high}$ Tregs correlated with the number of PMN-MDSCs. Thus, we concluded that there is no evidence that CIN MDSCs display impaired capacity to induce Tregs. In contrary, a role for PMN-MDSCs in the *in vivo* expansion of Tregs in our patients may be assumed, as these cells may induce normally Treg proliferation. Obviously, production of MDSCs is impaired/blocked in CIN in a different manner than the production of Tregs, which seems to be normal. Tregs are induced by several pathways besides MDSCs and are elevated maybe as a compensatory mechanism to suppress the inflammatory process within patients' BM and as a compensatory mechanism to their impaired production of MDSCs.

At this point, it should be noted that the population of both PB samples and BM samples is a small part of the wider study of the Laboratory and the small number may affect the results, which may differ in our final population. Regarding the measurement of our cell populations in healthy BMs, this became impossible due to the inability to find samples during the COVID19 pandemic period. The need for more data from healthy BM samples seems to be a more general problem in laboratories studying immune cells. These samples are difficult to find as BM biopsy is an invasive procedure without indication in healthy individuals. This makes it difficult to determine the normal range of the population of these cells, and to easily draw conclusions for our by patients. The results of this study and in particular the percentages of the studied cells in the BMMCs fraction of patients with CIN should be compared with the corresponding normal percentages. This will be done in the next period by the team of the Haemopoiesis Research Laboratory of the Medical School of the University of Crete with samples of "healthy" BMs from patients who undergo orthopedic surgery and do not have hematological disease. It remains to be seen whether the final results of the study will confirm our hypothesis.

Additionally, in order to draw safe conclusions about the ability of MDSCs to induce Tregs we need to undergo functional assays and prove the proliferation/induction of Tregs in cultures of MDSCs. Also, CIN Tregs as well as CIN MDSCs should be characterized more precisely with several functional markers, i.e. through isolation via cell sorting and RNA sequencing. These experiments are currently carried out by our team in our laboratory.

In conclusion, the results of this study, despite the fact that there are still several additional steps for final safe conclusions, are a strong indication that MDSCs as well as Tregs participate in the pathophysiology of CIN by reducing the numbers of MDSCs in the PB and as a compensatory mechanism with accumulation of PMN-MDSCs in patients' BM and increase of Tregs in patients' PB. Therefore, in the future, these cells could additionally contribute to clinical practice as biomarkers of disease severity and / or as therapeutic targets where treatment is needed, especially in patients with severe disease and frequent infections.

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