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The role of peripheral immunological tolerance in the pathogenesis of Systemic Lupus Erythematosus

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The role of peripheral immunological tolerance in the pathogenesis of Systemic Lupus Erythematosus

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To my daughter Evelina...

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1. ABSTRACT

Despite the complexity of the etiopathogenesis of autoimmune diseases, the great variability on clinical symptoms and the differential response of patients to existing therapies, there is a common denominator in autoimmunity; the loss of self tolerance. Therefore it appears necessary to delineate the causative mechanisms involved in the failure of the maintenance of self tolerance. Understanding the underlying mechanisms may offer the opportunity to develop targeted, timely and more effective therapies with benefits for a broader spectrum of autoimmune diseases. Myeloid-derived suppressor cells (MDSCs) is a heterogeneous population of immature myeloid cells with immunoregulatory properties that have recently gained interest in the field of autoimmunity. They comprise two subsets, the granulocytic and monocytic (G-MDSCs and M-MDSCs respectively) that can both suppress T cell responses.

In this project we focused on the role of MDSCs in systemic lupus erythematosus (SLE). SLE is the prototypic systemic autoimmune disease, where loss of tolerance results in chronic inflammation. Thus, we decided to examine the role of MDSC regulatory compartment in SLE, using the murine model NZB/W F1 that spontaneously develops lupus-like disease.

In this study we demonstrate for the first time an impaired expansion of $CD11b^{high}Gr\text{-}1^{+}$ MDSCs in NZB/W F1 lupus-prone mice, that is mainly attributed to $CD11b^{high}Ly6G^{+}$ G-MDSCs decreased levels in the bone marrow and in the peripheral lymphoid organs of lupus mice. The decreased levels were accompanied by an impaired function of $CD11b^{high}Ly6G^{+}$ G-MDSCs in lupus-prone mice; of note lupus G-MDSCs not only failed to suppress $CD4^{+}$ T cells *in vitro*, but actually promoted their proliferation and activation. Importantly, we found that $CD11b^{high}Ly6G^{+}$ G-MDSCs elimination was due

to increased extracellular trap formation (ETosis) in lupus mice driven by the inflammatory environment of lupus. Enhanced ET formation by G-MDSCs was dependent on reactive oxygen speces (ROS) production that was also induced by the inflammatory milieu of lupus. Moreover, the cytokines IFN-α, IFN-γ and IL-6 that are enriched in SLE serum were also identified as potential triggers of ETosis in $Ly6G^+$ granulocytes.

Overall, our data demonstrate a defective expansion of G-MDSCs in lupus microenvironment through the formation of ETs, a novel type of cell death. Importantly, we provide evidence for a ROS-mediated induction of ETs by $Ly6G^+$ granulocytes upon exposure to lupus inflammatory environment. Elimination of G-MDSCs may result in defective regulation of immune responses thus leading in disease progression. Our findings provide new insights into the pathogenetic mechanisms of SLE that could be exploited for therapeutic purposes.

1. Περίληψη

Παρά την πολυπλοκότητα της αιτιοπαθογένειας των αυτοάνοσων νοσημάτων, της πλούσιας ποικιλίας των κλινικών συμπτωμάτων μεταξύ ασθενών και τη διαφορετική ανταπόκριση των ασθενών σε κοινές συμπτωματικές θεραπείες, υπάρχει μια κοινή αρχή: τα αυτοάνοσα νοσήματα αναπτύσσονται εξαιτίας της απώλειας της ανοσολογικής ανοχής στα αυτοαντιγόνα. Συνεπώς είναι αναγκαίο να διαλευκάνουμε τις γενεσιουργές αιτίες στις οποίες οφείλεται η αδυναμία ελέγχου και διατήρησης της ανοσολογικής ανοχής. Η κατανόηση των σχετικών υπεύθυνων μοριακών μηχανισμών θα προσφέρει την ευκαιρία ανάπτυξης στοχευμένων, έγκαιρων και αποτελεσματικότερων θεραπειών με κοινά οφέλη για ένα μεγάλο εύρος αυτοάνοσων νοσημάτων. Τα κατασταλτικά κύτταρα μυελικής προέλευσης (MDSCs) είναι ένα ετερογενής πληθυσμός μυελικών, ατελώς διαφοροποιημένων κυττάρων με ανοσορυθμιστικές ιδιότητες, τα οποία πρόσφατα έχουν συγκεντρώσει το ενδιαφέρον της επιστημονικής κοινότητας. Αποτελούνται από δύο υποπληθυσμούς κυττάρων, τα κοκκιοκυτταρικά (G-MDSCs) και τα μονοκυτταρικά (M-MDSCs). Το κύριο χαρακτηριστικό των MDSCs είναι ότι μπορούν να καταστείλουν τις ανοσολογικές αποκρίσεις των Τ λεμφοκυττάρων.

Στην παρούσα ερευνητική εργασία, εστιάσαμε στο ρόλο των MDSCs στην παθογένεια του Συστηματικού Ερυθηματώδη Λύκου (ΣΕΛ). Ο ΣΕΛ είναι ένα συστεμικό αυτοάνοσο νόσημα, όπου η επιμένουσα απώλεια των ρυθμιστικών μηχανισμών διατήρησης της ανοσολογικής ανοχής συμβάλει στην παθογένεια της ασθένειας και την εγκαθίδρυση χρόνιας φλεγμονής. Για το λόγο αυτό, σχεδιάσαμε να εξετάσουμε το ρόλο των MDSC ρυθμιστικών κυττάρων στο ΣΕΛ, χρησιμοποιώντας το ζωικό μοντέλο NZB/W F1, ποντίκια τα οποία αυθόρμητα αναπτύσσουν ένα φαινότυπο παρόμοιο με του ΣΕΛ στον άνθρωπο.

Με την εργασία αυτή δείχνουμε πως τα CD11b^{high}Gr-1^{+} MDSCs ανιχνεύονται σε χαμηλά επίπεδα στα NZB/W F1 ποντίκια, τόσο στο υποκλινικό όσο και στο κλινικό στάδιο της νόσου. Αυτό κυρίως οφείλεται στη μειωμένη συχνότητα εμφάνισης των CD11b^{high}Ly6G^{+} G-MDSCs στο μυελό των οστών και στα περιφερικά λεμφικά όργανα των ποντικιών με τη νόσο. Ο μειωμένος αριθμός των CD11b^{high}Ly6G^{+} G-MDSC συνοδεύονται και από την ελαττωματική λειτουργία τους όσον αφορά στην *in vitro* καταστολή των CD4^{+} Τ λεμφοκυττάρων. Ενδιαφέρον προκαλεί ότι τα G-MDSC του λύκου όχι μόνο δεν μπορούν να καταστείλουν τα CD4^{+} Τ λεμφοκύτταρα, αλλά αντίθετα προκαλούν τον πολλαπλασιασμό κα την ενεργοποίηση τους. Σημαντικό είναι το εύρημα μας ότι τα CD11b^{high}Ly6G^{+} G-MDSCs είναι μειωμένα στο λύκο εξαιτίας της αυξημένης απελευθέρωσης εξωκυττάριων παγίδων (Extracellular Traps, ETs) που προκαλείται από το φλεγμονώδες περιβάλλον του λύκου. Η αυξημένη απελευθέρωση των ETs (ΕΤωση) εξαρτάται από την παραγωγή αντιδραστικών ριζών οξυγόνου (Reactive Oxygen Speces, ROS), η οποία επίσης προάγεται από τις φλεγμονώδεις ιδιότητες του περιβάλλοντος του λύκου. Τέλος, δείχνουμε ότι τρεις από τις εμπλουτισμένες κυτταροκίνες στο περιβάλλον του λύκου, οι IFN-α, IFN-γ και IL-6 έχουν την ικανότητα να επάγουν την ΕΤωση στα κοκκιοκυτταρικά κύτταρα.

Συμπερασματικά, τα δεδομένα μας αποδεικνύουν τη μειωμένη έκπτυξη των G-MDSC κυττάρων στο μικροπεριβάλλον του λύκου εξαιτίας του σχηματισμού των ETs, μια διαδικασία που αποτελεί ένα καινοφανή τύπο κυτταρικού θανάτου. Επιπλέον, παρέχουμε στοιχεία που υποστηρίζουν τη μεσολάβηση των ROS στο φαινόμενο αυτό. Η εξάλειψη των G-MDSC ενδεχομένως να έχει ως αποτέλεσμα την ελαττωματική ρύθμιση των ανοσολογικών αποκρίσεων συμβάλλοντας έτσι στην εξέλιξη της ασθένειας. Τα ευρήματά μας παρέχουν νέα στοιχεία στην κατανόηση της παθογένειας του ΣΕΛ και θα μπορούσε να συνεισφέρει στην ανάπτυξη νέων θεραπευτικών μεθόδων.

2. INTRODUCTION

Immune system

The immune system consists of specialized cells and molecules that all together in coordination respond against all invasive infectious agents in order to eliminate any pathogenicity. Such infectious agents are any bacteria, viruses, fungi and large multi-cellular parasites that manage to perturb the host's immune system homeostasis. In general, the immune defense mechanisms consist of two systems; the innate and the adaptive immune system. The innate immune system forms the first line of defense and is made up of several components, such as the epithelial barriers, that prevent pathogens entry in the body; specialized cells, the phagocytes, that destroy the pathogens that manage to invade; and natural antibiotics such as the complement and chemokines that help pathogens elimination. Apart from generally eliminating any pathogen, another important feature of innate immune system is that it can enhance adaptive immune responses against infectious agents that resist innate immunity. The adaptive immune system is comprised of B lymphocytes that produce antibodies and constitute its humoral part and T lymphocytes, the cellular part of adaptive responses that either helps B lymphocytes produce antibodies (T helper cells) or directly mediates a cytotoxic response against pathogens or cells attacked by pathogens (T cytotoxic cells). The adaptive immune system differs from the innate in that lymphocytes express specialized receptors on their surface that recognize an enormous variety of different substances produced by pathogens, named as antigens. The ability of both B and T cell receptors (BCR and TCR respectively) to recognize diverse antigens of potentially unlimited specificity relies on the somatic recombination of variable (V), diversity (D), and joining (J) gene segments that code for the variable regions of the receptors. This somatic recombination is mediated by a group of enzymes called the VDJ recombinase, composed of the recombinase-activating gene

(RAG)-1 and RAG-2 proteins. Another unique characteristic of the adaptive immune system is memory, meaning that each time a host is challenged with a pathogen that has been recognized in the past, the immune response against it is specific, immediate and efficient due to the action of memory lymphocytes that have been produced during the first invasion of the same pathogen.

Collectively, the physiologic function of the immune system is to act against pathogens in order to eliminate them; however, in certain situations, other substances that are noninfectious elicit immune responses as well. One such situation is autoimmunity.

Autoimmunity

Autoimmunity describes the reactions of an organism against its own (self) cells and tissues. It is estimated that approximately 5 % of the human population is affected by an autoimmune disease (Marrack, Kappler et al. 2001). Normally the immune system of an organism is unresponsive to self antigens; this unresponsiveness is called immunological tolerance and is maintained through a variety of mechanisms that facilitate the immune system to discriminate between self and nonself antigens. A failure of an organism to maintain/control immunological tolerance leads to the development of autoimmune diseases and subsequent tissue damage and organ failure. According to the clinical symptoms, autoimmune diseases can be classified into two types; organ-specific, where the expression of autoimmunity is restricted to specific organs and systemic, where multiple organs are affected and usually become chronic. Typical examples of organ-specific autoimmune diseases are Hashimoto's thyroiditis, type 1 diabetes mellitus and multiple sclerosis and for systemic autoimmune diseases systemic lupus erythematosus (SLE), rheumatoid arthritis and

scleroderma. In systemic autoimmune diseases the autoantigens are abundant and located in all cells, in multiple organs, as for example in SLE where the DNA, chromatin proteins and ribonucleoproteins can all serve as autoantigens. Despite extensive studies in this field the exact etiology of autoimmune diseases is far from being understood due to their complexity. Several genes as well as environmental factors and their interplay contribute to the development of an autoimmune disease but their exact role remains elusive.

Immunological Tolerance to Self Antigens

During normal lymphocyte development, the mechanisms by which lymphocyte receptors are expressed are not inherently biased to produce receptors for nonself antigens. Therefore, lymphocytes with the ability to recognize self antigens are constantly being generated. The ability of the immune system to discriminate between self and nonself relies on tolerogenic mechanisms, the functionality of which prevents immune responses against self antigens. Immunological tolerance to different self antigens may occur when the developing lymphocytes encounter these antigens in the central lymphoid organs, namely the bone marrow and thymus (central tolerance) or when the already mature lymphocytes encounter self antigens in peripheral tissues (peripheral tolerance).

Mechanisms of Central and Peripheral T cell Tolerance

Central Tolerance

The main mechanisms of central tolerance are deletion, change of function, editing and anergy.

Deletion

During T lymphocytes maturation in the thymus, many immature lymphocytes that recognize a self antigen with high avidity are deleted (negative selection) via the apoptotic pathway. Deletion occurs in case a self antigen is found in the thymus in high concentration or self peptide-MHC complexes are specifically recognized by T cells with high affinity. Deletion of double-positive T cells takes place in the thymic cortex and of single-positive T cells in the medulla. Similarly, immature B lymphocytes that recognize self antigens with high affinity in the bone marrow die by apoptosis. Exceptionally, if the reactivity between a self antigen and a B lymphocyte is weaker, this may lead to functional inactivation (anergy) rather than deletion.

Differentiation into regulatory T cells

Some self reactive $CD4^+$ lymphocytes that recognize self antigens in the thymus differentiate into regulatory T cells instead of being deleted. These regulatory cells leave the thymus and participate in peripheral tolerance as it will be discussed below.

Editing

As an alternative to deletion, immature self-reactive B lymphocytes can change their specificity by reactivating RAG1 and RAG2 genes and expressing a new immunoglobulin (Ig) light chain. This process is called receptor editing and eliminates self-reactivity from the mature B cell repertoire.

Peripheral tolerance

Although different tolerogenic mechanisms are active in the bone marrow, still some autoreactive lymphocytes reach the periphery. There, immune cells are subjected into

additional control and self-reactivity is limited due to the peripheral mechanisms of tolerance, namely the anergy, deletion and suppression by regulatory T cells.

Anergy

Mature T cells that are exposed to a self antigen in peripheral tissues in the absence of costimulation or innate immunity become functionally unresponsive, a process called anergy. The suggested mechanisms of anergy are the blockage of TCR-induced signal transduction, proteolytic degradation of the TCR complex proteins after ubiquitination or inhibition of TCR signaling after engagement of the inhibitory receptors of CD28 family, such as cytotoxic T lymphocyte antigen (CTLA)-4 and programmed cell death (PD)-1. Similar mechanism of anergy apples for B cells as well. The anergic B cells appear incapable of activating receptor-associated tyrosine kinases or maintaining sustained increases in intracellular calcium on exposure to the antigen

Deletion

Another mechanism of T cell tolerance following recognition of self antigens without inflammation or repetitive stimulation in the periphery is deletion by apoptotic cell death. Activation of a mature T cell in the absence of co-stimulation and growth factors activates effector proteins that in turn trigger apoptotic cell death via the mitochondrial pathway. One such protein is Bim pro-apoptotic protein, member of the B cell lymphoma (Bcl)-2 family. Repeated stimulation of T cells results in the co-expression of death receptors and their ligands, such as Fas and Fas ligand (FasL), that in turn activate intracellular cysteine proteases, named caspases, which trigger apoptotic death. As with T cells, B cells that encounter self antigens in the periphery have decreased survival and die through activation of the mitochondrial pathway, which is activated by the increased levels of Bim protein.

Follicular exclusion

Additionally to the mechanism described above, when mature B cells encounter self antigens in the peripheral tissues they lose their ability to migrate into the lymphoid follicles. This likely happens when chronic recognition of an antigen leads to reduced expression of the chemokine receptor CXCR5 that is essential for B cell migration into the follicles. As a consequence, self-reactive B cells are excluded from the follicles and stay in the periphery where they do not receive the necessary survival signals and fatally die.

Suppression by regulatory T cells

Regulatory T cells (Tregs) are able to suppress immune responses and thus serve to maintain self tolerance. As it was described above, regulatory T cell are generated in the thymus (naturally occuring Tregs) but they can also develop in the periphery (adaptive Tregs). The majority of this $CD4^+$ regulatory cell compartment express high levels of the interleukin-2 (IL-2) receptor α chain (CD25), but no other markers of activation. Another important molecule that is critical for $CD4^+CD25^+$ Tregs development and function is the transcription factor FoxP3, member of the forkhead family of transcription factors. Although the exact mechanisms by which Tregs control immune responses are not fully understood yet, it is known that Tregs secrete immunosuppressive cytokines such as IL-10, which inhibits the function of macrophages and dendritic cells. Moreover, Tregs functionality relies on the transforming growth factor (TGF)-β secretion, which inhibits the responses of lymphocytes and macrophages. Alternatively, regulatory T cells may also act by directly interacting with and suppressing other lymphocytes or antigen presenting cells. $CD4^+CD25^+$ FoxP3 expressing T cells are the most common Tregs but not the only one; there are other populations that also exert immunosuppressive mechanisms, such as the T_H3 and T_R1 cells that both lack CD25 expression.

Systemic Lupus Erythematosus (SLE)

SLE, a chronic, remitting and relapsing disease, is the prototypic systemic autoimmune disease and affects several vital organs and tissues such as the kidney, skin, lungs, brain and heart (Bertsias, Salmon et al. 2010; Tsokos 2011). The reported prevalence of SLE ranges from 20 to 150 cases per 100,000 population and it affects women (before menopause) nine times more often than men (Ref Understanding the epidemiology of SLE) which shows that the disease is also affected by the female hormones. The development of the disease depends both on genetic and environmental factors and its progression is dominated by the formation of immune complexes that lead to a variety of clinical manifestations such as rash, nephritis, glomerulonephritis, proteinuria, seizures, arthritis, thrombocytopenia, serositis and psychosis (Podolska, Biermann et al. 2015). The long lasting intent on delineating the pathogenic causes of SLE has led to the identification of several key mechanisms of the immune system that contribute to the pathogenesis of the disease.

SLE Pathogenesis

The main substrates that serve as autoantigens in SLE are the nucleic acids, nucleosomes, ribonucleoproteins and nucleolar antigens. The central source of these autoantigens is thought to be the apoptotic bodies, that in SLE are inefficiently cleared due to either increased apoptosis and/or defected/inefficient clearance of apoptotic cell debris. The continuation of apoptosis and inefficient clearance results in the persistence of nuclear antigens that subsequently leads to the production of autoantibodies by autoreactive B cells with the help of autoreactive T cells and myeloid dendritic cells (mDCs) or follicular DCs (fDCs). Opsonization of these circulating autoantigens by the respective autoantibodies results the formation of inflammatory immune complexes and uptake of the necrotic material by phagocytes

and plasmacytoid DCs (pDCs), the natural interferon (IFN)-producing cells. The increased IFN-α production leads to an overexpression of IFN-α-regulated genes, shaping the so-called “type-1 interferon signature” in patients with SLE. As a consequence $CD4^+$ autoreactive T cells expand, via CD80 and CD86 upregulation, and this supports autoreactive B cell and $CD8^+$ cytotoxic T cell survival. Moreover, IFN-α upregulates TLR7 which in turn enhances the responses against immune complexes containing nucleic acids and elevates the production of IFN-α, thus fueling an inflammatory loop (Ganguly, Chamilos et al. 2009). The formation of immune complexes is a key player in the pathogenesis of lupus. Immune complexes are mainly composed mainly of self DNA and nucleoprotein antigens and specific antibodies. They are deposited in the blood vessels and in the kidneys and initiate Fc receptor- and complement- mediated inflammatory cascade that eventually cause tissue injury, as proinflammatory cytokines are released and inflammatory cells are recruited to the location. Simultaneously, the DCs mature and secrete type I IFN and present the self antigens to the infiltrating T cells which undergo further activation. In this way adaptive immune responses are promoted; Th2, Th1, Th3, Th17 and B cell responses are amplified and in turn activate a new wave of effector cells, such as monocytes and polymorphonuclear cells (PMNs); a series of events that finally enforces a vicious circle of immunogenic pathways that contributes to the establishment of chronic inflammation.

Cytokine disturbances in SLE

Cytokines play a key role in the pathogenesis of SLE and have pleiotropic effects in the regulation of systemic inflammation, tissue damage and immunomodulation. Collectively, the cytokines that are mostly implicated in the disease pathogenesis are type I IFNs, IFN-γ, IL-6, TNF-α, IL-10, B-lymphocyte stimulator, IL-2, IL-21 and IL-17

(Pascual, Farkas et al. 2006; Rahman and Isenberg 2008; Ohl and Tenbrock 2011). In the scope of this study, only the role of type I IFNs, IFN-γ and IL-6 will be further discussed.

Type I IFNs

Almost all SLE patients display elevated levels of type I IFN in the serum. Peripheral blood mononuclear cells (PBMCs) from all active pediatric and most adult SLE patients display a remarkable IFN signature (Baechler, Batliwalla et al. 2003; Bennett, Palucka et al. 2003). One important finding is that IFN-α might break peripheral tolerance through activation and maturation of myeloid DCs (Blanco, Palucka et al. 2001). In SLE, the IFN-mediated maturation of DCs drives the activation of cytotoxic $CD8^+$ T cells to generate nucleosomes that can be engulfed and presented by DCs generated in the presence of interferon (Blanco, Pitard et al. 2005). Type I IFNs might also act directly on B cells by enhancing autoantibody secretion (Le Bon, Schiavoni et al. 2001) and T cells by promoting their survival (Marrack, Kappler et al. 1999) and their differentiation in effector and memory cells (Kolumam, Thomas et al. 2005). Another study by Liu Z *et al*. in NZB/ W lupus-prone mice shows that administration IFN-α in mice renders them relatively resistant to therapeutic intervention (Liu, Bethunaickan et al. 2011). The pleiotropic effects of IFN-α on immune cells could explain the breakdown of tolerance to nuclear antigens, autoantibody secretion and IC formation in SLE (Pascual, Farkas et al. 2006).

IFN-γ

In general, IFN-γ can activate macrophages at the site of inflammation, contributes to the activation of cytotoxic T cells, has antiviral properties and drives Th1 responses

(Ohl and Tenbrock 2011). Studies of the role of this cytokine in NZB/W F1 mice has shown that IFN-γ correlates with the survival, disease severity and perpetuation, progression of glomerulonephritis as well as serum levels of anti-dsDNA antibodies (Jacob, van der Meide et al. 1987; Haas, Ryffel et al. 1998; Enghard, Langnickel et al. 2006). Several studies in SLE patients show that IFN-γ serum levels are elevated and correlate with disease activity (Uhm, Na et al. 2003) and that it promotes Th17 responses (Shah, Lee et al. 2010), but its role in the pathogenesis of lupus is complex and remains controversial.

IL-6

IL-6 is produced by many cell types and has various biological activities, such as the differentiation of B lymphocytes in plasma cells that produce IgG (Muraguchi, Hirano et al. 1988), the differentiation and proliferation of T cells (Lotz, Jirik et al. 1988) and macrophages (Sachs, Lotem et al. 1989). The contribution of IL-6 in the pathogenesis of lupus is supported by several studies in Mrl/lpr and NZB/W mouse models. Studies show that IL-6 deficiency is correlated with delayed onset of lupus nephritis and prolonged survival and that IL-6 promotes IgG antibody production, the development of disease and acceleration of glomerulonephritis (Finck, Chan et al. 1994; Mihara, Takagi et al. 1998; Cash, Relle et al. 2010). Additionally, in active SLE patients the serum levels of IL-6 are elevated and are correlated with disease activity (Linker-Israeli, Deans et al. 1991) and other manifestations of the disease such as anti-DNA levels and proliferative nephritis (Grondal, Gunnarsson et al. 2000; Tackey, Lipsky et al. 2004). Overall, IL-6 exerts systemic effects and the delineation of its role in the pathogenesis of SLE gains interest.

Animal models in SLE

Due to the complexity of SLE disease and the numerous clinical manifestations observed, it would have been impossible to explore the underlying pathogenetic mechanisms in humans. Besides, due to ethical limitations in experimenting with human samples, research towards this direction has been facilitated by the use of animal models, especially murine. There is a variety of animal models for SLE. The vast majority develop autoantibodies such as antinuclear and anti-DNA antibodies, but also renal disease, in particular glomerulonephritis; these manifestations are the most commonly used for measuring the primary outcome of disease development in experimental studies. Other lupus-like features that mouse models develop are dermatitis, arthritis, cytopenias, vasculitis and neurological manifestations. The selection of the correct mouse model should be based on the area of interest and other factors, such as the end-organ disease manifestations, the non-lupus-like traits that are often exhibited, the timeframe and the existing knowledge (Perl 2012). There are two major groups of mouse models, the spontaneous and the experimentally induced models.

Spontaneous models

There is a variety of spontaneous models, that are genetically predisposed to develop lupus-like disease, however we refer to the most commonly used in the field.

New Zealand Black/White (NZB/W) F1 hybrid mice

This hybrid is considered by many researchers to resemble the human disease most closely (Dixon, Andrews et al. 1978; Singh, La Cava et al. 2007). It derives from the crossing of New Zealand Black (NZB) females with New Zealand White (NZW) males.

NZB mice display B cell hyperactivity, thymic loss, fatal autoimmune hemolytic anemia due to anti-erythrocyte antibodies and sometimes mild glomerulonephritis (Howie and Helyer 1968). In contrast, NZW are clinically healthy, but confer lupus in the NZB/W F1 hybrid; therefore they possess genetic determinants of the disease and profoundly efficient regulatory genes that allow this true-bred strain to appear normal. The female NZB/W F1 hybrid develops autoantibodies, including anti-nuclear and anti-DNA antibodies which correlate with the development of proliferative glomerulonephritis, associated with polyclonal B cell activation and T cell-dependent autoantibody production (Perl 2012).

New Zealand Mixed (NZM) mice

This strain has been established by recombinant inbreeding of the NZB/W F1 progeny. It develops more rapid and severe glomerulonephritis than NZB/W F1 (Morel, Rudofsky et al. 1994). They also display other traits such as Coombs positive anemia due to antierythrocyte antibodies and neurologic deficits. It is proposed that the NZM strains, particularly those with reduced disease penetrance or partial genotypes, provide an improved genetic model for assessment of the effects of environmental agents on SLE and autoimmunity (Rudofsky and Lawrence 1999).

MRL/Fas^{lpr} mice

The MRL/Fas^{lpr} carries a mutation in the apoptosis-related gene CD95 (FasR) that arose spontaneously during inbreeding of the MRL/Mp strain (Dixon, Andrews et al. 1978; Watanabe-Fukunaga, Brannan et al. 1992). This strain develops lymphoproliferation and lethal glomerulonephritis, but also has elevated hallmark serological markers. Additionally, it displays cognitive and affective dysfunction which

make it an appropriate model for the neuropsychiatric SLE (Gulinello and Putterman 2011).

BXSB mice

BXSB mice derive from SB/Le and C57BL/6 strains, develop lupus-like disease characterized by glomerulonephritis, antinuclear and anti-DNA antibodies, B cell hyperactivity and T cell-dependent autoantibody production (Andrews, Eisenberg et al. 1978; Dixon, Andrews et al. 1978). They differ from other strains, in that males are more susceptible than females, due to a mutation they carry on the Y chromosome, known as Yaa (Y chromosome-linked autoimmune accelerator), (Izui, Ibnou-Zekri et al. 2000).

Experimentally Induced Models

Different manipulations in certain mouse strains have successfully produced lupus-like phenotypes. Such models are the hydrocarbon oil (pristane), the chronic GVHD, the 16/6 idiotype, the bacillus Calmette-Guerrin (BCG) injection and some genetically targeted animals (Perl 2012). Below, we discuss on the pristane-induced model which is commonly used and resembles well the lupus-like autoimmunity in several strains.

Hydrocarbon oil (pristane)

Mice are injected intraperitoneally with pristane and the induced autoimmune phenotype is characterized by autoantibody production, an immune-complex glomerulonephritis and late-onset arthritis. It is considered as a good model of environmentally induced lupus (Satoh and Reeves 1994). This model has the

advantages being simple and reproducible and thus it is useful for assessing the role of novel therapeutic strategies.

Myeloid-Derived Suppressor Cells

The immune system shows an amazing range of complex mechanisms that facilitate immune regulation and tolerance and new information in this field is constantly gained. Recently, a newly-identified cell population with regulatory properties has gained interest; the Myeloid-Derived Suppressor Cells (MDSCs). This term has been suggested in 2007 by Gabrilovich *et al*. and reflects the origin and the biologic function of these cells that started being identified in 1970s – 1980s (Gabrilovich, Bronte et al. 2007). MDSCs are a heterogeneous population of immature myeloid cells that mediate immune suppression, as they have a remarkable ability to suppress T-cell responses. They contain myeloid precursors of granulocytes, monocytes/ macrophages, and dendritic cells. In healthy individuals, immature myeloid cells comprise ~0.5% of peripheral blood mononuclear cells. In contract, in pathological conditions such as cancer, various infectious diseases, sepsis, transplantation or autoimmune disorders, MDSCs are activated and expanded (Gabrilovich and Nagaraj 2009).

Origin, subsets, phenotype and localization of MDSCs.

MDSCs reside in the bone marrow. During hematopoiesis in the bone marrow, the hematopoietic stem cells (HSCs) differentiate into common myeloid precursor cells (CMPs). CMPs can generate immature myeloid cells (IMCs), as part of normal process of myelopoiesis. In healthy individuals, IMCs migrate to different peripheral organs and quickly differentiate into mature granulocytes, macrophages or dendritic

cells. Notably, MDSCs are a heterogeneous population of activated IMCs that have been prevented from fully differentiating into mature cells, are expanded in pathological conditions and exert suppressive activities (Gabrilovich 2004; Gabrilovich and Nagaraj 2009).

In mice, MDSCs are defined as cells expressing both the myeloid lineage differentiation antigen Gr-1 and CD11b (a_M-integrin). Gr-1^+CD$11b^+$ MDSCs lack the expression of cell-surface markers that are specific for monocytes, macrophages or dendritic cells (Bronte, Wang et al. 1998; Gabrilovich, Ishida et al. 1998; Kusmartsev, Nefedova et al. 2004). Normally, in the bone marrow MDSCs represent 20-30% of total cells, while in spleen they make up only a small proportion (2-4%) of splenocytes and are totally absent from lymph nodes (Gabrilovich and Nagaraj 2009). The Gr-1 epitope is shared by two molecules; Ly6G and Ly6C. Total MDSCs are characterized by differential expression of Gr-1, that reflects the existence of different subpopulations. Indeed, the establishment of Ly6G- and Ly6C- specific monoclonal antibodies, in combination with observations of MDSC morphology, has led to the identification of two MDSC subsets in tumor-bearing mice; the granulocytic MDSCs (G-MDCs) characterized as CD$11b^+$Ly$6G^+$Ly$6C^{low}$ and the monocytic (M-MDSCs) characterized as CD$11b^+$Ly$6G^-$Ly$6C^{high}$ (Hestdal, Ruscetti et al. 1991; Movahedi, Guilliams et al. 2008; Sawanobori, Ueha et al. 2008; Youn, Nagaraj et al. 2008). G-MDSCs and M-MDSCs differ phenotypically but share a common biologic activity, this of immune suppression. However, the two subsets utilize different molecular mechanisms to suppress T cell function and their role under different pathological conditions varies a lot (Dietlin, Hofman et al. 2007; Zhu, Bando et al. 2007; Movahedi, Guilliams et al. 2008; Youn, Nagaraj et al. 2008).

In humans, MDSCs are most commonly defined as $CD14^{-}CD11b^{+}$ cells that also express the common myeloid marker CD33 but lack the expression of markers of mature myeloid or lymphoid cells and the MHC-class II molecule HLA-DR (Ochoa, Zea et al. 2007). MDSCs have also been identified within a $CD15^{+}$ population in human peripheral blood (Schmielau and Finn 2001). Several studies have identified G-MDSCs ($CD11b^{+}CD14^{-}CD15^{+}$), (Zea, Rodriguez et al. 2005) and M-MDSCs ($CD11b^{+}CD14^{+}HLA\text{-}DR^{low/-}$), (Filipazzi, Valenti et al. 2007). MDSCs from patients with renal cell cancer, express markers of activated granulocytes, including high levels of CD66b and low levels of CD62L, CD16 and vascular endothelial growth factor (VEGF) receptor 1 (Rodriguez, Ernstoff et al. 2009; Nagaraj and Gabrilovich 2010; Peranzoni, Zilio et al. 2010; Ribechini, Greifenberg et al. 2010). It is apparent that the absence of universal markers for MDSC characterization in humans renders their identification difficult. Therefore, further investigation of human MDSCs is required.

Expansion and activation of MDSCs

Recently, a “two-signal” model has been suggested to describe the accumulation of MDSCs under pathological conditions. This model suggests that MDSCs expansion depends on two processes that are governed by two different signal transduction pathways and are responsible initially for the expansion of MDSCs and subsequently their activation (Condamine and Gabrilovich 2011). According to this model, the process of expansion is induced by various cytokines and growth factors, while the second activating signaling pathway requires pro-inflammatory molecules. However, it is likely that there is an overlap in these two signaling pathways (Condamine and Gabrilovich 2011). Analytically the molecules that are involved in each process are discussed below.

MDSCs expansion is induced by cytokines and growth factors that are produced by tumors or bone marrow stroma in response to chronic stimulation. Such factors are granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF) and stem-cell factor (SCF). During MDSC expansion, the signaling pathways that are activated by these molecules signal primarily via signal transducer and activator of transcription 3 and 5 (STAT3 and STAT5), which are signaling molecules that are involved in cell survival, proliferation, differentiation and apoptosis (Bromberg 2002). This signaling prevents differentiation of MDSC and promotes proliferation of immature myeloid cells (Condamine and Gabrilovich 2011). In tumor bearing mice, ablation of STAT3 expression in conditional knockout mice or selective STAT3 inhibitors markedly reduced the expansion of MDSCs and increased T-cell responses (Nefedova, Huang et al. 2004; Kortylewski, Kujawski et al. 2005). The target molecules that are activated by STAT3 and are associated with increased survival and proliferation of myeloid progenitors include B-cell lymphoma XL (BCL-XL), cyclin D1, MYC and surviving (Condamine and Gabrilovich 2011). Moreover, STAT3 induces the expression of S100A8 and S100A9, members the family of S100 calcium binding proteins, which have been reported to have an important role in inflammation (Foell, Wittkowski et al. 2007). STAT3-dependent upregulation of S100A8 and S100A9 expression by myeloid progenitor cells prevented their differentiation and resulted in the expression of MDSCs in the spleen of tumor-bearing and naive S100A9-transgenic mice (Cheng, Corzo et al. 2008). Additionally, S100A8 and S100A9 have been implicated in MDSCs migration to the tumor site in tumor-bearing mice (Sinha, Okoro et al. 2008). All together these proteins contribute to proliferation and survival of immature myeloid cells and prevent their differentiation to mature cells, leading to the consequent expansion of cells with the phenotype of MDSC.

As mentioned above the accumulation of MDSCs requires a second activating signal besides expansion. In cancer, this signaling is provided by activated T cells and tumor stromal cells that produce factors such as IFNγ, ligands for Toll-like receptors (TLRs), IL-13, IL-4, IL-1β and transforming growth factor-β (TGFβ). In turn, these molecules activate several different signaling pathways in MDSCs that involve STAT6, STAT1 and nuclear factor-κB (NF-κB) (Condamine and Gabrilovich 2011). STAT6 is activated by the binding of IL-4 or IL-13 to the receptor IL-4Rα and leads to MDSC activation. Experiments have shown that STAT6 deficiency prevents signaling downstream IL4-Ra and thereby blocks the production of arginase 1 (ARG1) by MDSCs (Sinha, Clements et al. 2005). STAT1 is mainly activated by IFN-γ and is responsible for *ARG1* and induced nitric oxide synthase (*iNOS*) expression (Gabrilovich and Nagaraj 2009). When IFN-γ that is produced by activated T cells is blocked, MDSC-mediated T cell suppression is abolished (Kusmartsev and Gabrilovich 2005; Movahedi, Guilliams et al. 2008). Moreover, MDSCs from $Stat1^{-/-}$ mice failed to upregulate *ARG1* and *iNOS* expression and did not inhibit T cell responses (Kusmartsev and Gabrilovich 2005). In MDSCs, the TLR family plays a prominent role in NF-κB activation, primarily via the myeloid differentiation primary response gene (MyD88). Consistently, MDSCs accumulate and are activated during microbial and viral infections, as well as in trauma and sepsis (Condamine and Gabrilovich 2011). NF-κB, acting downstream of MyD88, is required for accumulation of MDSCs in a model of polymicrobial sepsis (Delano, Scumpia et al. 2007) and LPS, and in combination with IFN-γ could promote MDSC expansion, probably by inhibiting differentiation of DCs (Greifenberg, Ribechini et al. 2009). STAT1 is also activated by IL-1β. In transgenic mice that expressed human IL-1β specifically in their stomach, the resulted spontaneous gastric inflammation and cancer, correlated with early recruitment of MDSCs to the stomach, while the use of IL-1β antagonist inhibited the development of gastric preneoplasia and suppressed MDSC mobilization (Tu, Bhagat et al. 2008).

In vitro differentiation of MDSCs

Due to MDSCs strong potential to suppress immune responses, these cells have gained interest in the field of cell therapy of diseases such as autoimmunity, asthma, organ transplantations and graft versus host disease (GVHD). For the use of MDSC for therapeutic intervention it would be necessary to be able to generate these cells *in vitro*. There are recent studies that deal with several factors that are necessary to drive MDSC expansion and differentiation *ex vivo*. Indicatively, treatment of bone marrow progenitor cells with LPS and IFN-γ, in the presence of GM-CSF, shifted their differentiation from DC to MDSC (Greifenberg, Ribechini et al. 2009). In other studies, the combination of GM-CSF with IL-6 or G-CSF was sufficient to drive MDSC expansion (Lechner, Liebertz et al. 2010; Marigo, Bosio et al. 2010). GM-CSF alone or in combination with IL-1β, IL-6, TNF-α, or VEGF resulted the differentiation of $CD33^+$ MDSCs from PBMCs isolated from a healthy donor (Lechner, Liebertz et al. 2010). Moreover, GM-CSF and G-CSF, in combination with IL-13 induced preferential differentiation of M-MDSC which were suppressive through an arginase mechanism in GVHD (Highfill, Rodriguez et al. 2010). Conclusively, it is apparent that the generation of MDSCs from undifferentiated cells is feasible and their clinical utility remains to be verified in order to improve cell therapy.

Mechanisms implicated in MDSC suppressive activity

There are several suppressive mechanisms that MDSCs utilize to regulate immune responses that apply either to both G-MDSCs and M-MDSCs or specifically to one of each. Some mechanisms require cell-to-cell contact while others only soluble mediators. The most well characterized mechanisms include arginase, iNOS, reactive oxygen species (ROS) and peroxynitrite or by inducing T regulatory (Treg) cells.

Arginase and iNOs are enzymes secreted by MDSCs that catalyze L-arginine, an essential amino acid for the regulation of T cell proliferation (Rodriguez, Hernandez et al. 2005; Ochoa, Zea et al. 2007). Arginase converts L-arginine into urea and L-ornithine; the consequent shortage of L-arginine inhibits T-cell proliferation through different mechanisms including decrease of CD3ζ expression (Rodriguez, Zea et al. 2002) and halt of the cell cycle regulators expression such as cyclin D3 and cyclin-dependent kinase 4 (CDK4), (Rodriguez, Quiceno et al. 2007). The enzyme iNOS converts L-arginine into nitric oxide (NO) which suppresses T cell proliferation through inhibition of JAK3 and STAT5 (Bingisser, Tilbrook et al. 1998), or MHC class II (Harari and Liao 2004) and induction of T cell apoptosis (Rivoltini, Carrabba et al. 2002). Another key characteristic of MDSCs that correlates with their suppressive activity is high production of ROS. More than one studies in mice and cancer patients showed that inhibition of ROS production by MDSCs completely abrogated the suppressive effect on T cells *in vitro* (Schmielau and Finn 2001; Kusmartsev, Nefedova et al. 2004). Microbial products and inflammation are also known to induce development of a MDSC population that produces ROS and NO and modulates activated T cells expansion (Dietlin, Hofman et al. 2007). In EAE, $CD11b^{+}Ly\text{-}6C^{high}$ MDSCs suppressed T cell proliferation through IFN-γ-dependent NO production (Zhu, Bando et al. 2007).

Another mediator of MDSC suppressive activity is the peroxynitrite ($ONOO^{-}$), an oxidant that is produced by the chemical reaction between NO and superoxide anoion (O_2^{-}). Peroxynitrite induces the nitration and nitrosylation of the amino acids cystine, methionine, tryptophan and tyrosine (Vickers, MacMillan-Crow et al. 1999). In many cancer types high levels of peroxynitrite at the sites of MDSC and inflammatory cell accumulation are associated with tumor progression (Vickers, MacMillan-Crow et al. 1999; Ekmekcioglu, Ellerhorst et al. 2000; Cobbs, Whisenhunt et al. 2003; Nakamura, Yasuoka et al. 2006). Recent studies have shown that nitration of the molecules on

the surface of $CD8^+$ T cells, localized at the site of physical interaction of MDSCs with T cells, led to TCR complexes disruption and therefore Ag-specific $CD8^+$ T cell tolerance in cancer (Nagaraj, Schrum et al. 2010). This phenomenon of MDSC-induced antigen-specific T-cell unresponsiveness in tumor-bearing mice was also observed *in vivo (Kusmartsev and Gabrilovich 2005)*.

Apart from MDSCs direct role in suppressing T cells, different studies show MDSCs intermediate role in tolerance through the induction of T regulatory cells. Different studies indicate different mechanisms implicated in this procedure. In tumor-bearing hosts, the production of NO by $Gr\text{-}1^+CD115^+$ M-MDSC was required to suppress antigen-associated activation of tumour-specific T cells but was dispensable for Treg-cell induction (Huang, Pan et al. 2006). In a mouse model of B cell lymphoma, MDSCs were shown to induce expansion of Tregs through a mechanism that required arginase and the capture, processing and presentation of tumor-associated antigens by MDSCs (Serafini, Mgebroff et al. 2008). Another study with CD40-deficient mice suggested that CD40/CD40L interactions between M-MDSCs and Tregs play important role in the induction of the latter, as the deficient mice were unable to support tumor-specific T reg expansion (Pan, Ma et al. 2010).

The above mentioned suppressive mechanisms are not necessarily used equally by the different MDSC subsets. Indeed, G-MDSCs and M-MDSCs express different effector molecules. More specifically, in tumor bearing mice G-MDSCs were found to express high levels of ROS and low levels of NO, whereas the monocytic subset expressed low levels of ROS and high levels of NO. However both subsets expressed arginase (Youn, Nagaraj et al. 2008). Of interest the two populations were efficiently suppressive of T cell proliferation to an equal extent. Similarly, in another study with

tumor-bearing mice it was revealed that G-MDSCs suppressive activity was Arg1-dependent while M-MDSCs were dependent on STAT1 and iNOS. Despite the distinct effector mechanisms used, both subsets were able to suppress antigen-specific T cell responses (Movahedi, Guilliams et al. 2008). The exact biological significance of this diversity remains unclear but it sets it necessary to examine the role of the two subsets separately each time MDSCs are studied.

MDSCs in autoimmunity

The first studies of MDSCs come from the field of cancer. However, the study field of MDSCs has been broadened to other diseases such as bacterial and parasitic infections, acute and chronic inflammation, traumatic stress, surgical sepsis, transplantation and recently autoimmunity as well. In experimental autoimmune uveoretinitis, an animal model of human intraocular inflammatory disease, the number of MDSCs was significantly increased (Kerr, Raveney et al. 2008). In a murine model of type 1 diabetes, MDSCs could prevent diabetes onset and markedly decrease Ag specific autoimmune responses (Yin, Ma et al. 2010). Of note, G-MDSCs were shown to accumulate prior to disease remission and ameliorate disease when transferred *in vivo* in mice with Experimental Autoimmune Encephalomyelitis (EAE), a model of multiple sclerosis (Ioannou, Alissafi et al. 2012). Similarly, MDSCs have a regulatory role in rheumatoid arthritis, as Fujii W and colleagues showed that in collagen-induced arthritis model MDSCs accumulated in correlation with disease severity and could suppress $CD4^+$ T cell responses (Fujii, Ashihara et al. 2013). In the case of inflammatory bowel disease, studies in a murine colitis model have shown that MDSCs could suppress the proliferation of splenocytes in vitro and decreased intestinal inflammation when transferred *in vivo (Guan, Moreno et al. 2013)*. All these reports exemplified the emerging role of MDSCs in autoimmunity as a critical

regulatory compartment for the development of inflammation. However, the exact role of MDSCs and the underlying mechanisms remain unclear and it is important to focus our efforts on delineating them and evaluate MDSCs potential to serve as a therapeutic target.

Neutrophils

Neutrophils, together with basophils and eosinophils belong to the granulocyte family of white blood cells, named polymorphonuclear cells (PMNs). PMNs are characterized by a uniquely segmented lobular nucleus and the existence of cytoplasmic granules filled with degradative enzymes. Neutrophils are the most abundant type of circulating white blood cells and they are the major cell type mediating acute inflammatory responses to microbial infections. Neutrophils are characterized by a short time life span in the circulation in order to limit excessive inflammatory responses. However, their lifespan can be increased in response to cytokines or other proinflammatory agents (El Kebir and Filep 2013).

One hallmark of PMNs is the presence of cytoplamsic granules, special storage organelles that contain bactericidal substances. Neutrophils contain four types of granules, the azurophilic (primary), the specific (secondary), the gelatinase (tertiary) and the secretory vesicles. The azurophilc are the first to be formed during neutrophil maturation and contain myeloperoxidase (MPO), defensins, lysozyme, bactericidal/permeability-increasing protein (BPI), and a number of serine proteases such as neutrophil elastase (NE), proteinase 3 (PR3) and cathepsin G (CG), (Nusse and Lindau 1988; Faurschou and Borregaard 2003; Lacy 2005). The specific granules are characterized by the presence of the glycoprotein lactoferrin, but also contain a wide range of antimicrobial compounds including NGAL, hCAP-18, and lysozyme

(Faurschou and Borregaard 2003; Lacy 2005). The third type ,the gelatinase granules, contain a few antimicrobials, but they serve as a storage location for a number of metalloproteases, such as gelatinase and leukolysin (Borregaard 2010). In contrast to the three classical types of granules mentioned above, the secretory vesicles cargo consists of plasma-derived proteins such as albumin and their membrane proteins facilitate neutrophil migration (Borregaard, Sorensen et al. 2007).

Neutrophils are produced during myelopoiesis in the bone marrow where growth factors and cytokines drive the differentiation of pluripotent hematopoietic progenitors into neutrophils. During this process, granules are simultaneously formed in the cytoplasm of neutrophils (Borregaard 2010). In response to infection, the production of neutrophils increases rapidly, a procedure that is mainly orchestrated by granulocyte-colony stimulating factor (G-CSF), (Lieschke, Grail et al. 1994). Retention and release of neutrophils from the bone marrow is determined by the balance between Chemokine (C-X-C Motif) Receptor (CXCR) 4 (favoring retention) and CXCR2 (favoring release) and their ligands stromal cell-derived factor 1 (SDF-1), and chemokine (C-X-C motif) ligand (CXCL)-1 and CXCL2, respectively (Eash, Means et al. 2009; Eash, Greenbaum et al. 2010). G-CSF stimulates neutrophil release directly by effects on the neutrophil and indirectly by reducing the stromal cell-derived factor 1 (SDF-1) expression and enhancing the expression of CXCL2 on endothelial cells (Christopher, Liu et al. 2009). Neutrophils are able to migrate from the blood stream to extravascular sites of infection based on a well-orchestrated procedure characterized by a selectin-mediated rolling, an integrin-mediated firm adhesion and a chemokine-mediated motility through the endothelium towards the site of inflammation (Amulic, Cazalet et al. 2012).

Once neutrophils have traversed the endothelium, they are exposed to a different inflammatory milieu composed of chemoattractants that derive from the host and inflammatory stimulants of pathogenic origin. Thus, new signaling pathways are activated and neutrophils movement towards the site of inflammation, where danger signals are at an ever-higher concentration, is based on the chemoattractants gradient. One key host-derived neutrophil recruiting chemokine and activator is IL-8 that successively stimulates cell migration, then initiates oxidative burst and finally induces degranulation (Ley 2002). The pathogen-associated stimuli are collectively known as pathogen-associated molecular patterns (PAMPs) and include LPS, bacterial lipopeptides, flagellin and DNA. PAMPs are recognized by neutrophil pattern-recognition receptors that are either membranous or intracellular. The best known receptors are Toll-like receptors (TLRs), (Parker, Whyte et al. 2005), but others exist as well such as receptors for formyl methionine peptids, cytokine receptors such as IFNγ receptros (IFNγR), mannose receptors, scavenger receptors and receptors for products of complement activation and antibodies (Abbas, Lichtman et al. 2010; Amulic, Cazalet et al. 2012).

Finally, neutrophil activation is followed by the execution of microbial killing which is facilitated by three main functions; 1) phagocytosis, 2) degranulation and 3) neutrophil extracellular trap formation (NETosis). Phagocytosis is the procedure by which pathogen particles are internalized by the cell membrane into vacuoles called phagosomes. In the case of neutrophils, phagosomes mature upon fusion with granules a critical step that allows the delivery of antimicrobial substances inside the phagosomal lumen. Simultaneously, the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is assembled on the phagosomal membrane resulting in the production of reactive oxygen species (ROS) that are toxic for the pathogens. The sustained NADPH oxidase activity is essential for maintaining an alkaline pH in the

phagosomes that in turn allows for the activation of the major serine proteases neutrophil elastase (NE) and cathepsin G (CG), (Lee, Harrison et al. 2003).

During degranulation the content of neutrophil granules is released at the inflammatory site and produces an environment inhospitable to invading pathogens. To date, any observations of degranulation process come from biochemical approaches performed *in vitro*. The technical limitations restrict our knowledge on the exact mechanism of degranulation *in vivo*, but it assumed that the release of granular components could occur primarily through other means, most notably through formation of neutrophil extracellular traps, cell damage or cell lysis (Amulic, Cazalet et al. 2012).

Neutrophil Extracellular Trap formation (NETosis)

NETosis is the third function that neutrophils exert in order to eliminate pathogens. It is a recently identified, novel cell death mechanism with antimicrobial benefits against neutrophils life. Neutrophil Extracellular Traps (NETs) have been characterized as web-like structures, released by neutrophils in the extracellular milieu, that are composed of decondensed chromatin decorated by granule and cytoplasmic proteins (Brinkmann, Reichard et al. 2004).

What triggers NETosis

Many physiological inducers of NETosis have been reported. Infections with bacteria, fungi, and HIV parasites induce NETs. Other physiologically relevant stimuli are ROS, like hydrogen peroxide (Fuchs, Abed et al. 2007). NET formation is also triggered,

albeit inefficiently, by antibodies (Kessenbrock, Krumbholz et al. 2009) and antibody–antigen complexes (Garcia-Romo, Caielli et al. 2011; Lande, Ganguly et al. 2011), and by microbial components such as lipopolysaccharide (Neeli, Dwivedi et al. 2009; Lim, Kuiper et al. 2011), M1 from *Streptococcus pyogenes (Oehmcke, Morgelin et al. 2009)*, or lipophosphoglycans from *Leishmania amazonensis (Guimaraes-Costa, Nascimento et al. 2009)*. Rapid NET formation is also induced by platelets activated via TLR-4 (Clark, Ma et al. 2007). Moreover, nitric oxide was recently demonstrated to induce NETs in a process that was dependent on MPO (Patel, Kumar et al. 2010). NET formation appears to require attachment of neutrophils to a substrate that stimulates the MAC-1 integrin receptors (Neeli, Khan et al. 2008).

Mechanism of NET formation

During NET formation, the chromatin is decondensed and the nuclear material is expanded, thus leading to nuclear envelope disintegration. Consequently, the cytoplasmic membrane ruptures and NETs are liberated extracellularly (Fuchs, Abed et al. 2007). The decondensation of chromatin is correlated with the conversion of histone 3 (H3) arginine to citrulline residues, a process known as citrullination and performed by peptidylarginine deiminase (PAD4), (Wang, Wysocka et al. 2004). Citrullination must play central role in NETosis as PAD4-deficient mice failed to form NETs and were more susceptible to necrotizing fasciitis induced by group A *Staphylococci* than wild-type mice (Li, Li et al. 2010). Two other critical factors for NET formation are NE, a serine protease that is stored in azurophilic granules and contributes to antimicrobial activity in the phagosome and MPO that is also stored in azurophilic granules and is crucial for the production of toxic oxidants. As NETs are formed, NE translocates from the granules to the nucleus where it partially cleaves the histones to promote chromatin decondensation in synergy with MPO

(Papayannopoulos, Metzler et al. 2010). Moreover, ROS production through NADPH oxidase activity, as well as autophagy are required for NET release when neutrophils are stimulated with the ROS agonist phorbol myristate acetate (PMA), (Remijsen, Vanden Berghe et al. 2011). This observation is strengthened by the fact that patients with chronic granulomatosus disease (CGD), who are deficient in NADPH oxidase activity are susceptible to opportunistic infections, particularly to fungal pathogens (Bianchi, Hakkim et al. 2009). Upstream of NADPH, the activation of Raf-MEK-ERK pathway is required for NET formation (Hakkim, Fuchs et al. 2011).

In most studies in the field, NETosis is accompanied by neutrophil cell death. However, there are alternative processes that have been reported and describe nuclear fragments and chromatin release without plasma disintegration (Pilsczek, Salina et al. 2010). Of note, Yousefi *et al*. suggested that GM-CSF-primed neutrophils expel DNA, associated with granule proteins, that is generated by the mitochondria while neutrophils remain alive (Yousefi, Mihalache et al. 2009).

NETs are decorated with intracellular proteins most of which are of granulocyte origin, fewer are nuclear and even more rare are the cytoplasmic, as it was identified by mass spectrometry (Urban, Ermert et al. 2009). NET proteins are primarily the cationic (thus, DNA-binding) bactericidal proteins: histones, defensins, elastase, proteinase 3, heparin binding protein, cathepsin G, lactoferrin, and myeloperoxidase (Urban, Ermert et al. 2009), but also the pattern recognition molecule Pentraxin 3 (Jaillon, Peri et al. 2007) and the S100A8,9 complex, known as calprotectin (Murthy, Lehrer et al. 1993) are NET associated. The proteins that decorate NETs, together with DNA, physiologically serve as a means to trap pathogens and eliminate them. However, the proteins that are found on NETs can often be immunogenic as NETs constitute a rich

pool of self antigens, at the same time. Especially when NET clearance is defective, the protein context of NETs can drive autoimmunity, as it will be discussed below.

NETs clearance

As the inflammation resolves NETs are removed in a process where the serum DNase1 has prominent role (von Kockritz-Blickwede, Chow et al. 2009; Hakkim, Furnrohr et al. 2010). The debris left by DNase1 activity are possibly cleared by phagocytes, macrophages and neutrophils that are newly recruited at the inflammatory site (Bratton and Henson 2011).

Extracellular Trap Formation

Of interest, neutrophils are not the only PMNs that release extracellular traps (ETs). More specifically, eosinophils and mast cells also release ETs that are composed of DNA and antmicrobial proteins (von Kockritz-Blickwede, Goldmann et al. 2008; Yousefi, Gold et al. 2008). Notably, a distinct subset of proinflammatory cells, the low-density granulocytes (LDGs) was shown to release ETs that promote autoantigen externalization and organ damage in SLE (Carmona-Rivera and Kaplan 2013). Actually, these LDGs display phenotypic characteristics of immature neutrophils with non-segmented nuclei and higher expression of MPO, NE, and defensin-3, and they may be related to MDSCs (Hacbarth and Kajdacsy-Balla 1986; Bennett, Palucka et al. 2003). However, no official study has been yet referred to ETosis by MDSCs. Additionally, extracellular trap formation (ETosis) has been reported in monocytes and macrophages, to a lesser extent compared to neutrophils however (Bartneck, Keul et al. 2010; Webster, Daigneault et al. 2010).

NETosis in autoimmunity

NETosis is a principle antimicrobial mechanism involved in host defence against pathogens. However, recent data suggest that NETs play an important role in autoimmune diseases as well, such as in systemic vasculitides (Kessenbrock, Krumbholz et al. 2009; Sangaletti, Tripodo et al. 2012), rheumatoid arthritis (Liu, Tangsombatvisit et al. 2012; Khandpur, Carmona-Rivera et al. 2013) and systemic lupus erythematosus (Hakkim, Furnrohr et al. 2010; Villanueva, Yalavarthi et al. 2011).

NETs can be immunogenic in multiple ways. One of these is the exposure of autoantigens on their surface that can be targeted by immune cells in autoimmune diseases. A few representative examples of autoantigens exposed by NETs are dsDNA and histones for SLE, MPO and PR3 for ANCA-associated vasculitis and vimentin, enolase and histones for RA (Grayson and Kaplan 2016). Moreover, it has been reported that NETs can interact with APCs meriting autoantibody production. Of interest a study has shown that myeloid DCs upload netting components preferentially than contents from apoptotic or necrotic neutrophils (Sangaletti, Tripodo et al. 2012). Netting neutrophils can also activate B cells by inducing Ig class-switching and antibody production (Puga, Cols et al. 2012). Additionally, NETs have been shown to be involved in cell-to-cell interaction with T cells which results in increase of T cell responses to specifc antigens, through reduction of the activation threshold (Tillack, Breiden et al. 2012). Another immunogenic property of NETs that can mediate autoimmune responses is that they can trigger type I IFN secretion by pDCs. Relevant processes for NET-mediated Type I IFN production have been reported in SLE (Garcia-Romo, Caielli et al. 2011; Lande, Ganguly et al. 2011; Villanueva, Yalavarthi et al. 2011), psoriasis (Skrzeczynska-Moncznik, Wlodarczyk et al. 2012;

Skrzeczynska-Moncznik, Wlodarczyk et al. 2013) and type I dabetes (Diana, Simoni et al. 2013). Alternative immunogenic mechanisms orchestrated by NETs are the inflammasome machinery activation (Kahlenberg, Carmona-Rivera et al. 2013) and complement activation (Leffler, Martin et al. 2012). All these finding support the notion that persistent and deregulated NET formation may play an important role in autoimmune diseases initiation and perpetuation.

Especially for SLE, recent evidence implicates aberrant NET formation and impaired degradation in disease pathogenesis. In particular low density granulocytes (LDGs) isolated from lupus patients appear to be primed to form NETs *ex vivo* in the absence of additional stimuli. LDGs were shown to release dsDNA and inflammatory cytokines, while enhanced NET release in the periphery and in tissues was correlated with increased circulating ant-dsDNA titers (Villanueva, Yalavarthi et al. 2011). Another study has shown that immune complexes containing autoantibodies against antimicrobial peptides, such as LL37 and neutrophil peptides are deposited on NETs, thus blocking self-DNA degradation by nucleases and subsequently promoting their uptake by pDCs through TLR-9 activation. Activated pDCs release IFN-α which can further prime NET production (Lande, Ganguly et al. 2011). At the same time, NET degradation by DNase I appears impaired in one-third of SLE patients (Hakkim, Furnrohr et al. 2010). In correlation with impaired NET clearance, SLE patients have elevated levels of anti-nuclear and anti-NET antibodies, as well as higher prevalence of lupus nephritis and complement activation that in turn impairs NET degradation (Leffler, Martin et al. 2012). Last but not least, another report has demonstrated that NETs and NET-derived LL37 stimulate the NLP3 inflammasome machinery in lupus macrophages which may further promote proinflammatory responses in various organs, including kidneys and vasculature, through IL-18 and IL-1β effects (Kahlenberg, Carmona-Rivera et al. 2013). Increased NET formation has been

associated to lupus pathogenesis in several murine models as well. However, the exact role of NETosis in SLE pathogenesis is not completely defined so far; therefore further investigation in this field appears necessary.

3. OBJECTIVES

The principle aim of this project was to delineate the role of MDSCs in systemic lupus erythematosus. More specifically, we initially set the following objectives

- **Characterize MDSCs phenotype in central and peripheral lymphoid organs of lupus prone mice.**
- **Assess MDSCs functional properties and suppressive ability in lupus prone mice.**
- **Delineate the underlying mechanisms of MDSC-mediated immune regulation in lupus prone mice.**

Elaboration of the first results obtained pointed out the importance of formulating new objectives. Thus, we set the following aims;

- **Evaluate lupus CD11b^{high}Ly6G^{+} G-MDSCs potential to release extracellular traps (ETs).**
- **Investigate the causative mechanisms and the underlying molecular pathways that drive ETosis in lupus G-MDSCs.**
- **Identify molecules that mediate ETosis in G-MDSCs in the inflammatory environment of lupus.**

4. MATERIALS AND METHODS

Mice

Female NZB/W F1 hybrid mice, female NZB (6 mo old), male NZW (3 – 10 mo old), and C57BL/6 (B6) mice were obtained from the SPF animal facility of the Institute of Molecular Biology and Biotechnology (Heraklion Crete, Greece) and Harlan Laboratories (United Kingdom). Male NZB/W F1 hybrid mice were obtained from Jackson Laboratories (Bar Harbor ME, USA). All procedures were in accordance to institutional guidelines and were approved by the Greek Federal Veterinary Office.

Reagents

The fluorescent-conjugated monoclonal Abs Ly6G (1A8), Ly6C (HK1.4), CD11b (M1/70), Ly6G/Ly6C (GR-1) (RB6-8C5), CD11c (N418), CD4 (RM4-5), CD25 (pc61), CD44 (IM7), CD45R/B220 (RA3-6B2), IFN-αR-1 (MAR1-5A3), IFN-γR β chain (MOB-47), CD126 (IL-6Rα chain), (D7715A7) and rat IgG1k isotype control (RTK2071) were all from Biolegend. Cell viability was assessed with 7-AAD (BD Biosciences). Polyclonal anti-neutrophil elastase (anti-NE) Ab was from Abcam. Polyclonal anti-human myeloperoxidase (anti-MPO) was from Dako. Annexin V, purified anti-CD3 (145-2C11) and anti-CD28 (37.51) Abs were from BD Pharmingen. HRP-linked anti-rabbit Ab, Alexa Fluor 488 – labeled anti-rabbit Ab and CF555-conjugated goat anti-rabbit IgG (H+L) Ab were from Biotium. Collagenase D enzyme, DAPI dye and clinical sticks (Chemistrip 10 UA) were from Roche. Polyclonal anti-p62 Ab (SQSTM1) was from MBL International. Monoclonal anti-LC3 Ab was from Novus Biologicals. Murine rIL-6 and rIFN-γ were from Peprotech, INC, human rIFN-α (universal Type I IFN, rIFN-α) was from PBL. DMEM and RPMI culture medium, FBS, penicillin, streptomycin, 2-

mercaptoethanol, HEPES, BSA and PBS tablets were from Gibco. PureLink® RNA Mini Kit, TURBO DNA-free™ Kit were from Ambion. SuperScript™ First-Strand Synthesis System, Cell Trace CFSE Proliferation kit and HRP-linked anti-mouse Ab were from Invitrogen. iTaq™ Universal SYBR® Green Supermix was from BIO-RAD. RIPA cell lysis and protein extraction buffer was from Pierce. The monoclonal anti-actin Ab (clone C4) was from Millipore. Chemiluminescent Western detection reagent ECL was from GE Healthcare. DNase I enzyme, percoll, poly-L-lysine, Triton, PMA, CFA, 2', 7' Dichlorofluorescin diacetate (DCF) and Diphenyleneiodonium chloride (DPI) were from Sigma – Aldrich. N-acetyl-L-cysteine (NAC) was from Pharmazan. MitoSox reagent and Dynabeads Mouse T-Activator CD3/CD28 were from Life Technologies. The Protease Inhibitor Single – Use Cocktail was from Thermo Scientific.

Serum isolation

Blood serum was isolated from mouse peripheral blood, taken from the orbital sinus with the use of a glass Pasteur pipette. Blood samples were left at RT for 2 h and then kept overnight at 4°C. Blood was then centrifuged for 20 min, at maximum speed, at 4°C and supernatant was collected. The isolated serum was stored at –20°C.

Animal Immunization

Anaesthetized B6 (8 wk old) and NZB/W F1 pre-diseased (2 – 4 mo old) mice were immunized with 100μg MOG_{35-55} peptide (Genemed Synthesis Incorporation) or PBS respectively emulsified in CFA s.c. at the base of the tail (100 μl/mouse). Emulsions were prepared with the use of an ultrasonic homogenizer and were injected in mice with the use of a 1 ml syringe.

Single cell suspension preparation

Bone Marrow. To retrieve the BM cell suspensions the hind limbs were dissected away from the body of euthanized mice, by cutting above the femur with the help of scissors. Tissue from the limbs was removed with the help of the scissors before detaching the femur from the shinbone. Then, the proximal and distal ends of the bones were cut and with the help of a 10 ml syringe, filled with 5% PBS/ FBS. the bone marrow was flushed in a sterile petri dish. After flushing the bones in the petri dish, the tissue was homogenized by passing it through a $25G^{5/8}$ needle ten to twelve times. The single-cell suspension was then transferred in 5% PBS/ FBS medium, centrifuge for 10 min, at 400 g , at 4°C and the supernatant was discarded. The pellet was then resuspended and incubated in 2 ml NH_4Cl_2 (pH = 7.2 – 7.6) on ice to lyse the erythrocytes. The erythrolysis was stopped by adding 8 ml cold PBS 1x followed by centrifugation for 10 min, at 400 g, at room temperature (RT) to finally resuspend the pellet in 5% PBS/ FBS medium. Samples were kept at 4°C until further use.

Spleen. Spleen was extracted from mice with the use of scissors and placed in a 40 μm cell strainer, inside a petri dish containing 5 ml of 5% PBS/ FBS medium, on ice. The tissue was then homogenized with a syringe plunger to obtain single cell suspension. Cells were carefully transferred in a 15 ml falcon with a pipette and washed by centrifugation for 10 min, at 400 g, Erythrocytes were lysed with NH_4Cl_2 as described above. Spleenocytes were resuspended in 5% PBS/ FBS medium and kept at 4°C until further use.

Lymph nodes. The inguinal, branchial or axillary lymph nodes (LNs) were detach them from the mouse body with the help of a pair of forceps. Any fat remnants that could increase cell death were carefully clean away with the use of a pair of forceps.

LNs were then placed on 40 µm cell strainer in a petri dish, containing 5 ml of 5% PBS/ FBS medium. The tissues were smashed with the help of a syringe plunger on the cell strainer surface and cells suspensions were collected in a 15 ml falcon tube, filled up with 5% PBS/ FBS up to 15 ml. Samples were washed by centrifugation for 10 min, at 400 g, RT and supernatants were discarded. The pellets were resuspended in 1 ml of 5% PBS/ FBS and kept at 4°C until further use.

Flow cytometry and cell sorting

Single cell suspensions were prepared from tissues as described above and were stained for surface markers at 4°C, for 20 min in the dark, in a 5% FBS/ PBS medium. Cell acquisition and sorting was performed with DakoCytomation MoFloT High-Performance Cell Sorter and/or FACS Calibur (BD Biosciences). Dead cells were identified as 7-AAD$^-$ and excluded from acquisition analysis and sorted populations. Acquisition data were analyzed with FlowJo Software (Tree Star Inc.).

Ly6G$^+$ granulocytic cell isolation

Murine Ly6G$^+$ cells were isolated from total BM cell suspensions. The suspensions were placed on a discontinuous Percoll gradient (52%, 67%, 75%), (pH = 7,2 – 7,4) and centrifuged at 1000 g for 30 min without break. Cells from the 67% - 75% interface where PMNs lay were isolated, then washed with HEPES buffer (pH = 7,2 – 7,4) and re-suspended in culture medium. Purity of Ly6G$^+$ cells was assessed with FACS analysis and exceeded 80%.

Assessment and quantitation of ETosis

G-MDSCs or $Ly6G^+$ granulocytes were cultured on 10% poly-L-lysine-treated cover slides (VWR International) in RPMI medium (10 mM HEPES/2 % BSA) at 37°C, 5% CO_2 for 4 h. Cells were then fixed with 4% paraformaldehyde (20 min, RT) and then washed with PBS. The cover slides were then moved on parafilm cover surface and treated with 0,5% Triton/PBS (100 µl per cover slide) to achieve cell membrane permeability. Non-specific binding was blocked with a 30 min incubation with 5% BSA/PBS. To assess ETosis, cells were stained with anti-NE primary Ab for 1 h at RT, followed by 1 h incubation (RT) with Alexa-Fluor 488–labeled secondary Ab with intermediate washes with 5% BSA/ PBS. Cells were then stained with anti-MPO (1 h, RT), followed by incubation with CF555-conjugated secondary Ab, again with intermediate washes with 5% BSA/ PBS. DNA was labeled with DAPI (3 min, RT). Finally, the cover slides were mounted onto glass slides (Soft lab series) using mowiol for confocal microscopy (Leica SP2) as a mounting medium to avoid fade of the cover slides over the time. ETs were visualized by fluorescence microscopy and characterized as structures positively stained for NE, MPO and DNA (Papayannopoulos, Metzler et al. 2010). To determine the percentage of ET release, ET-forming cells were counted by microscopy observation (20x lens), (Olympus microscope System, BX61). For each sample five to ten randomly selected fields were analyzed and mean values were calculated. The mean of ET forming cells per mean total cells in the field was extrapolated in percentages.

Proteinuria assessment

Urine samples from NZB/W F1 mice were collected in metabolic cages where mice were left for at least 4 h while provided with drinking water. Proteinuria was assessed with a visual dipstick test. Sample protein content was graded as follows; negative,

traces, + (30 ng/dl), ++ (100 ng/dl), +++ (500 ng/dl). Mice with values from negative to + (30 ng/dl) were considered pre-diseased, while mice with more than ++ (100ng/dl) were considered diseased with established proteinuria.

Proliferation Assay

FACS-sorted $CD4^+CD25^-$ T cells isolated from the skin-draining and mesenteric LNs (mLNs) and 7-$AAD^-CD11c^-CD11b^{high}Gr\text{-}1^+$ MDSCs isolated from BM and spleen of NZB/W F1 or B6 CFA-injected mice were co-cultured in complete DMEM, at 1:1 ratio, in a round-bottom 96-well plate (Costar), for 5 d (37°C, 5% CO_2). The minimum number of each cell population was ideally $15x10^3$ cells. Before setting the co-culture, T cells were labeled with 1 μM CFSE in 0,1% BSA/ PBS medium (1 ml/10^6 cells) for 20 min at 37°C). CFSE-labeled T cells were polyclonally stimulated with 10 μg/ml plate bound purified anti-CD3 and 1 μg/ml soluble purified anti-CD28 or with Dynabeads Mouse T-Activator CD3/CD28 (1:1 ratio with T cells, according to manufacturer's protocol). Plate bounding with anti-CD3 required pretreatment of the plate wells the antibody at the respective concentration diluted in 50 μl PBS 1x and overnight incubation at 4°C or 3 h incubation at 37°C, followed by two washes with PBS 1x. At the end of the culture time, cell were retrieved from the plate with moderate pipetting and cells were stained with anti-CD4 and anti-CD25 with the protocol described above. Cell proliferation and activation of $CD4^+$ T cells were analyzed by FACs.

Quantification of ROS production levels

Cells (2×10^5/condition) were seeded onto a round bottom 96-well plate (Costar) in RPMI medium supplemented with 10 mM HEPES and 2% BSA and cultured for 1 h at

37°C, 5% CO_2. *Total ROS production.* PMA (100 nM) as positive control to induce oxidative stress, NAC (20 mM) as total ROS scavenger and DPI (20 μM) as inhibitor of NADPH oxidase activity, were used. Cells were cultured in the presence of 5 μM DCF to allow total ROS detection and analyzed by FACS, according to manufacturer's protocol. *Mitochondrial ROS production*. PMA (100 nM) and H_2O_2 (500 μM) were used to induce oxidative stress and ROS production. MitoSox reagent (5 μM) was used to quantify the production ROS specifically by mitochondria and samples were analyzed by FACS, according to manufacturer's protocol.

Statistical Analysis

Statistical analysis was performed with Prism (GraphPad Software). Student t test (two-tailed, 95% confidence interval) was used. *p* values less than 0.05 were considered statistically significant.

Real-time quantitative PCR

RNA was extracted from sorted MDSCs and treated with DNase. RNA concentration and purity was measured in NanoDrop 2000 spectrophotometer (Thermo Scientific). cDNA was synthesized and PCR was performed on CFX Connect™ Real-Time PCR Detection System (BIO-RAD) using SYBR green incorporation (20 μl reaction), according to manufacturer's protocol. The cDNA input was 15 ng per reaction. Reactions were performed with the following thermal profile: 95°C for 2 min, 40 cycles of 95°C for 15 sec and 58°C or 60°C for 30 sec, followed by melting curve protocol from 65°C to 95°C with 0.5°C increment for 5 sec each. Hprt was used as control. Gene relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method. The primers were

obtained from Invitrogen and used at the concentration of 400 nM each. The target genes and the respective primer sequences are shown in Table 1.

Table 1: Target genes primers sequence

Gene	Forward Primer	Reverse Primer
Cebpb	5'-ACGGGACTGACGCAACACAC-3'	5'-CCGCAGGAACATCTTTAAG-3'
Arg1	5'-CAGAAGAATGGAAGAGTCAG-3'	5'-CAGATATGCAGGGAGTCACC-3'
iNOS	5'-GTTCTCAGCCCAACAATACAAGA-3'	5'-GTGGACGGGTCGATGTCAC-3'
Hprt	5'-GTGAAACTGGAAAAGCCAAA-3'	5'-GGACGCAGCAACTGACAT-3'

Western Blot analysis

Cells (2 x 10^6 / condition) were lysed in RIPA lysis and extraction buffer (80 µl lysis buffer/ 2 x 10^6 cells) containing protease inhibitors. Proteins (40 µg/sample) were then separated by a 12% SDS-PAGE (100 – 150 V) and transferred (400 mA) for 90 min onto PVDF transfer membrane (Millipore). Overnight blocking at 4°C with 10% skimmed milk in TBST for p62 and 5% skimmed milk/ 1% BSA in TBST for LC3 preceded blotting of membranes with the according antibodies. Membranes were then blotted with polyclonal anti-p62 (1: 1000, in 1% skimmed milk in TBST) or monoclonal anti-LC3 (1:1000, in 5% skimmed milk/ 1% BSA in TBST). Afterwards, to allow detection upon usage of ECL reagent membranes were incubated with a secondary antibody, anti-rabbit (1:2000, in 5 % skimmed milk in TBST) that is linked to Horse Radish Peroxidase (HRP). Actin (1:5000, in 5% skimmed milk in TBST) was used as

loading control; detection was performed with the secondary anti-mouse (1:2000, in 5% skimmed milk in TBST). Protein binding was visualized by chemiluminescence detection at a ChemiDoc XRS^{+} images system and analyzed with the Image Lab Software (BIO-RAD). Actin protein was used as a loading control. Protein lysates from Neuro 2A cell line (Novus Biologicals) were used as positive control.

Collagenase/ DNase I treatment of kidneys

Kidneys were cut in small pieces and placed in complete DMEM medium supplemented with Collagenase D (0,4 mg/ml), DNase I (10^{-2} mg/ml) and 10% FBS (6 ml/kidney) at 37°C for 30 min with intermediate stirring. Cell suspensions were passed through a 40 μm cell strainer (FALCON), washed and re-suspended in 5% FBS/ PBS.

Annexin V – 7-AAD Apoptosis Assay

Cells (6 x 10^{5} /condition) were cultured in a 48-well plate (Costar) with DMEM medium supplemented with 2% FBS, NZB/W F1- or B6-derived serum, for 2 or 4 h. Cells were then stained with 7-AAD in 5% FBS/ PBS medium for 20 min at 4°C, followed by Annexin V staining, according to manufacturer's protocol. Cell viability was assessed by FACS.

5. RESULTS

5.1 Impaired expansion of CD11b^{high}Gr-1^{+} MDSCs in NZB/W F1 lupus-prone mice

The frequency of MDSCs is significantly increased in response to inflammation (Gabrilovich and Nagaraj 2009). In order to examine whether systemic autoimmune responses are accompanied by increased frequencies of CD11b^{high}Gr-1^{+} MDSCs we monitored the frequency of MDSCs in the BM and spleen of NZB/W F1 mice by flow cytometry. To analyze CD11b^{high}Gr-1^{+} frequency, cells were initially gated according to granularity (SSC) and size (FSC). Cell debris and dead cells were excluded by negatively selecting low FSC and high SSC cells. To further exclude dead cells from analysis we used 7-Aminoactinomycin D (7-AAD) viability stain according to 7-AAD potential to incorporate with compromised membranes. Thus, 7-AAD^{+} apoptotic/necrotic cells were excluded. Among live cells, MDSCs were characterized as CD11b^{high}Gr-1^{+} (**Fig. 1A and B left panels**) and frequencies among different groups were compared. According to previous studies, MDSCs expand in Experimental Autoimmune Encephalomyelitis (EAE) model that resembles multiple sclerosis in humans (Ioannou, Alissafi et al. 2012). Therefore we used EAE mice as a control experiment. Specifically, we injected B6 mice (8 wk old) with MOG/CFA and assessed MDSCs frequency in the BM and spleen, four to ten days post injection (p.i.). Figure 1 shows the dramatic expansion of MDSCs upon inflammation in EAE model. Interestingly, the frequency of CD11b^{high}Gr-1^{+} MDSCs was not significantly increased in BM of NZB/W F1 diseased mice with established proteinuria, in contrast to mice with EAE (**Fig. 1A right panel**). Furthermore, although CD11b^{high}Gr-1^{+} MDSC frequency was increased

in spleen of diseased compared to pre-diseased NZB/W F1 mice, their frequency was 2-3 fold decreased in NZB/W F1 mice compared to mice with EAE (**Fig. 1B right panel**). These data suggest a defective expansion of CD11b^{high}Gr-1^{+} MDSCs in the BM and spleen of lupus mice with established disease.

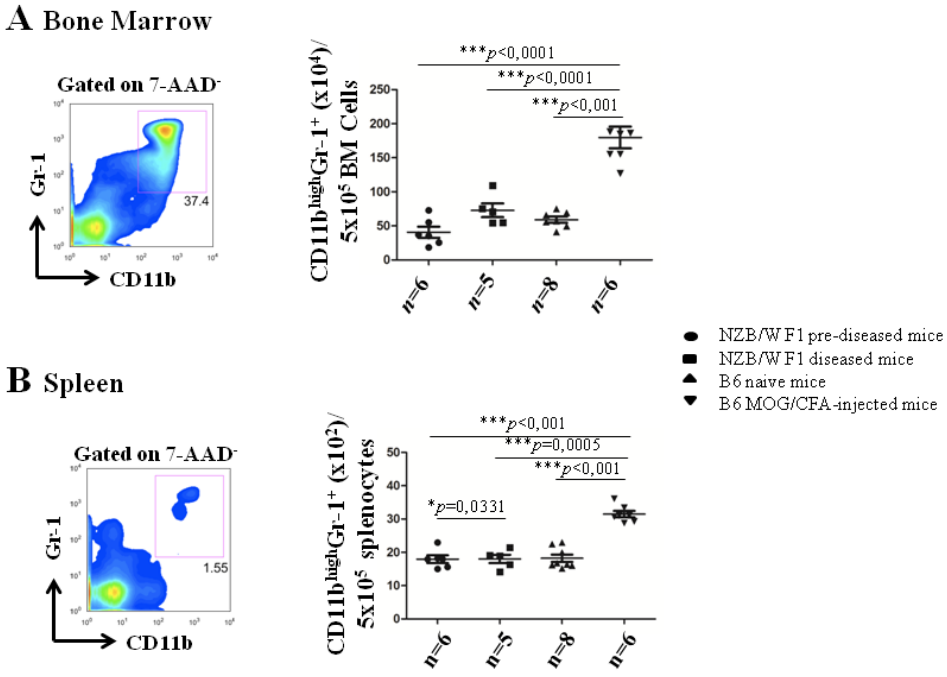


Figure 1. **Impaired expansion CD11b^{high}Gr-1^{+} MDSCs in NZB/W F1 lupus-prone mice**. **A** and **B**, Representative flow cytometric analysis and relative numbers of sorted CD11b^{high}Gr-1^{+} MDSCs/ 5 x 10^{5} total BM (**A**) and spleen (**B**) cells. Numbers in FACS plots denote frequency. Mean ± SEM is depicted.

5.2 CD11b^{high}Gr-1^{+} MDSCs expand upon immunization with CFA in NZB/W F1 mice

One possible reason why MDSCs expansion is defective in NZB/W F1 diseased mice could be that NZB/W F1 mice BM is incompetent and cannot mount an immune

response to inflammatory stimuli. To address this, we immunized NZB/W F1 pre-diseased mice (2-4 mo old) with CFA which is composed of inactivated and dried mycobacteria (*M. tuberculosis*). It is known that an immune response against this general stimulus is accompanied by systemic MDSCs expansion (Gabrilovich and Nagaraj 2009). We assessed MDSCs frequency 7 d p.i. and observed a significant expansion of MDSCs in CFA-challenged mice compared to NZB/W F1 non-immunized animals as shown by flow cytometry analysis and extrapolation of the results in MDSC frequency per $5x10^5$ BM cells (**Fig. 2A and B respectively**). These results indicate that the BM in NZB/W F1 mice is competent and able to respond to inflammatory stimuli.

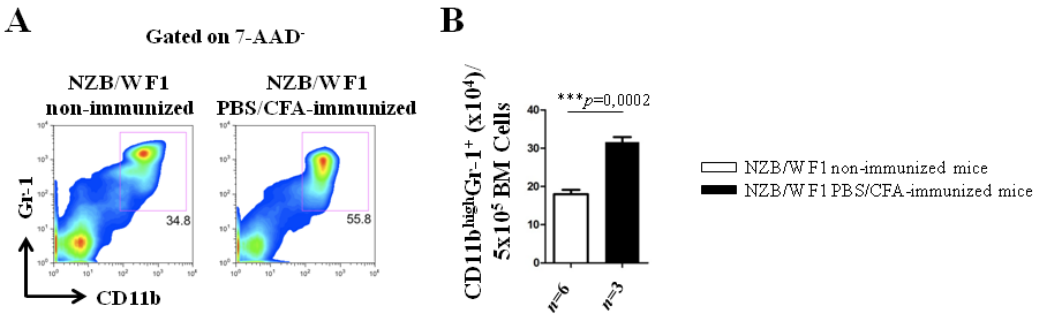


Figure 2. **Impaired expansion of MDSCs in NZB/W F1 mice could neither be attributed to incompetent BM. A**, Representative flow cytometric analysis of $CD11b^{high}Gr\text{-}1^+$ MDSCs in the BM of NZB/W F1 non-immunized and PBS/CFA-immunized mice. **B**, relative numbers of $CD11b^{high}Gr\text{-}1^+$ MDSCs/ 5 x 10^5 total BM cells.

5.3 $CD11b^{high}Gr\text{-}1^+$ MDSCs do not migrate to the inflamed tissues

Another property of MDSCs is to migrate at the inflammatory site (Gabrilovich and Nagaraj 2009). Thus, we reasoned that the absence of MDSCs from the periphery of NZB/W F1 mice with established proteinuria is due to migration to the inflamed kidneys or the renal LNs (rLNs). As shown in **Figure 3** no considerable accumulation

of CD11b^{high}Gr-1^{+} MDSCs was observed in kidneys or rLNs of NZB/W F1 diseased mice suggesting that the absence of MDSCs from the periphery could not be explained by their migration to the site of inflammation.

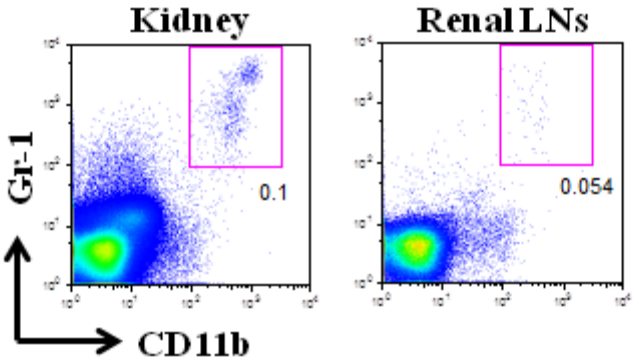


Figure 3. CD11b^{high}Gr-1^{+} MDSCs do not accumulate to inflamed target tissues in NZB/W F1 mice. Representative flow cytometric analysis of CD11b^{high}Gr-1^{+} MDSCs in the kidney and rLNs of diseased NZB/W F1 mice *n*=3 (two independent experiments). Numbers in FACS plots denote frequencies.

5.4 Impaired function of CD11b^{high}Gr-1^{+} MDSCs in NZB/W F1 lupus-prone mice

5.4.a. CD11b^{high}Gr-1^{+} MDSCs promote T cell responses in NZB/W F1 lupus-prone mice

The hallmark of MDSCs is their suppressive properties on T cell responses (Gabrilovich and Nagaraj 2009). Therefore, we sought to examine whether the function of CD11b^{high}Gr-1^{+} MDSCs is also compromised in NZB/W F1 mice. To answer this question, we assessed the *in vitro* proliferation of sorted T cells in the presence of syngenic MDSCs from NZB/W F1 pre-diseased mice. To eliminate any suppressive effect derived from regulatory cells we excluded CD25^{+} T cells from the culture system by isolating CD25^{-}CD4^{+} T cells (gated on 7-AAD^{-} events to exclude dead cells) from mesenteric and inguinal LNs (mLNs and iLNs) with FACS-sorting

(**Fig. 4A**). MDSCs were sorted according to the gating strategy described above plus that we ensured that the sorted population would contain no DCs, that could promote T cell responses, by gating CD11b^{high}Gr-1^{+} MDSCs on 7-AAD^{-}CD11c^{-} cells. CD11c^{+} DCs that were excluded from the MDSC population were separately used as a positive control. The effect of CD11c^{-}CD11b^{high}Gr-1^{+} MDSCs from NZB/W F1 pre-diseased mice was assessed on aCD3/aCD28-stimulated CFSE-labeled CD25^{-}CD4^{+} T cells, after having them in co-culture for 5 d. Surprisingly, lupus MDSCs not only failed to suppress but instead, promoted T cell activation and proliferation as evidenced by the CD44 expression and CFSE dilution respectively (**Fig. 4B**) similar to the effect of CD11c^{+} DCs.

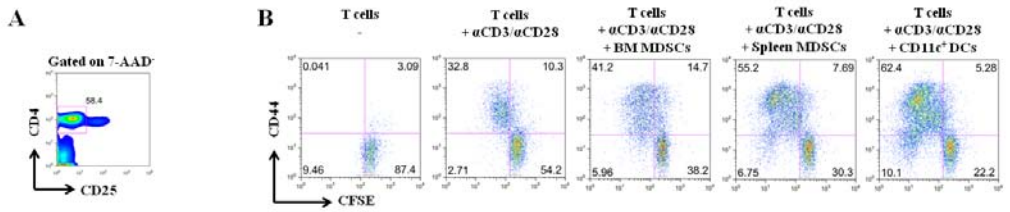


Figure 4. Defective function of CD11b^{high}Gr-1^{+} MDSCs in NZB/W F1 lupus-prone mice. **A,** Gating strategy for CD4^{+}CD25^{-} T cell sorting from mLNs of NZB/W F1 pre-diseased mice. **B,** Representative flow cytometric analysis of CD44 expression and CFSE dilution of CD4^{+} T cells stimulated by 10 μg/ml plate-bound αCD3 and 1 μg/ml soluble αCD28 and co-cultured with 5 x 10^{4} cells of the indicated purified cell populations at a 1:1 ratio. Representative results from four independent experiments are shown. **A** and **B,** Numbers in FACS plots denote frequencies.

5.4.b. Decreased *Cebpb, Arg1 and iNOS* gene expression in CD11b^{high}Gr-1^{+} MDSCs of NZB/W F1 lupus-prone mice

There are various molecules that have been implicated in the suppressive capacity of MDSCs such as *Cebpb* (Marigo, Bosio et al. 2010), arginase 1 (*Arg1*) and inducible

nitric oxide synthase (*iNOS*), (Bronte, Serafini et al. 2003). More precisely the immunoregulatory activity of MDSCs is entirely dependent on the Cebpb transcription factor, as it was shown by Marigo *et al*. (Marigo, Bosio et al. 2010). This group confirmed that complete loss of Cebpb with the use of *Cebpb*$^{flox/flox}$ mice resulted in full abrogation of BM-MDSC and tumor-derived MDSCs immunosuppressive activity on antigen-activated CD8^{+} T cells. Similarly, *Arg1* and *iNOs,* the products of which share L-arginine as a common substrate, play critical role for MDSCs immunosuppressive activity as it was supported by Bronte *et al*. who showed that arginase and iNOS inhibitors abrogated the inhibition of alloreactive T cells by MDSCs (Bronte, Serafini et al. 2003).

Therefore, we assessed *Cebpb, Arg1* and *iNOS* expression in CD11b^{high}Gr-1^{+} cells sorted from the BM of NZB/W F1 and EAE mice. We found significantly decreased *Cebpb* and minimal *Arg1* expression in BM CD11b^{high}Gr-1^{+} MDSCs of both pre-diseased and diseased NZB/W F1 compared to cells from B6 naive or EAE mice (**Fig. 5**) and undetectable expression of *iNOs* (data not shown). These results are in accordance with the defective suppressive activity of CD11b^{high}Gr-1^{+} MDSCs in NZB/W F1 lupus mice. Collectively, our findings indicate impaired expansion and aberrant function of CD11b^{high}Gr-1^{+} MDSCs in the course of SLE.

5.5 Impaired expansion of CD11b^{high}Gr-1^{+} MDSCs is attributed to decreased levels of CD11b^{high}Ly6-G^{+} G-MDSCs

MDSCs is a heterogeneous population consist of two cell subsets, G-MDSCs and M-MDSCs that utilize different effector mechanisms to suppress T cells and often respond differently in various diseases and inflammatory stimuli (Youn, Nagaraj et al. 2008). Thus, we sought to delineate whether both monocytic and granulocytic MDSC

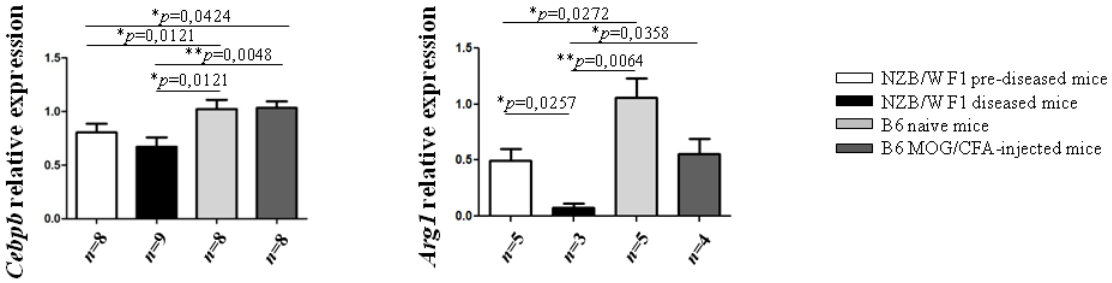


Figure 5. *Cebpb and Arg1* expression is down-regulated in NZB/W F1 mice. Relative quantitation of *Cebpb* and *Arg1* gene expression by RT-PCR in $CD11b^{high}Gr\text{-}1^{+}$ MDSCs sorted from indicated mouse groups, using the $2^{-\Delta\Delta Ct}$ method. Data shown are mean of two independent experiments, normalized to *HPRT* expression.

subsets are affected in NZB/W F1 lupus mice or not. Towards this direction we analyzed the two subsets frequency in the BM and spleen of pre-diseased and diseased NZB/W F1 mice by FACS analysis, using EAE model again as a disease control. All the cells that were included in the analysis were $7\text{-}AAD^{-}CD11c^{-}CD11b^{high}$; G-MDSCs were $Ly6G^{+}Ly6C^{-}$ and M-MDSCs were $Ly6G^{-}Ly6C^{+}$ (**Fig. 6A and B, left panels**). The results were reported as number of MDSCs per $5x10^{5}$ BM cells or splenocytes. Neither M-MDSC nor G-MDSC cells were expanded in BM of diseased NZB/W F1 mice (**Fig. 6A, right panels**). Interestingly, only G-MDSCs failed to expand in spleen of diseased NZB/W F1 mice whereas M-MDSCs were found significantly increased upon disease development (**Fig. 6B right panels**).

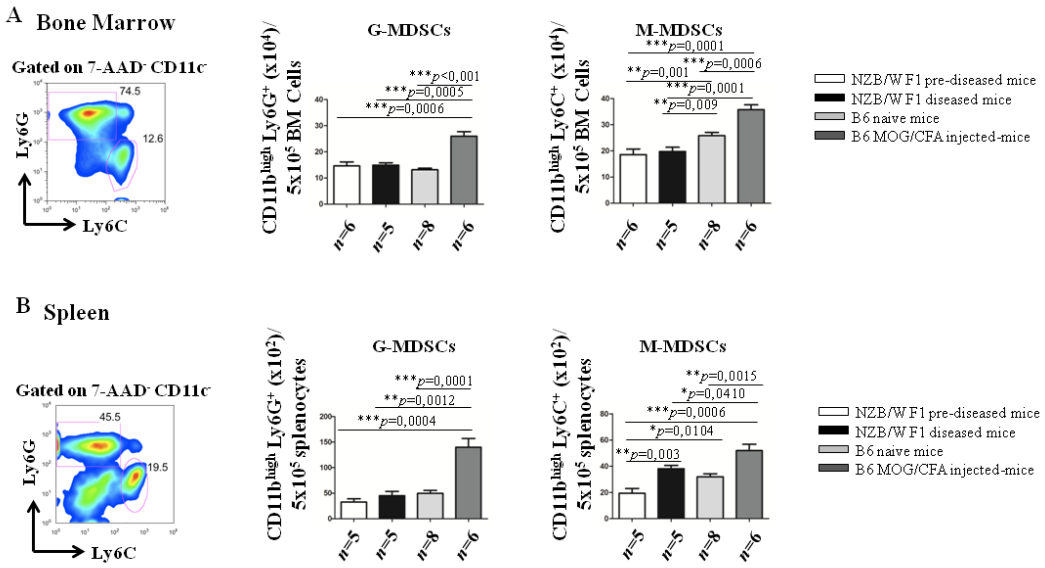


Figure 6. Defective expansion of G-MDSC compartment in NZB/W F1 mice. A and **B**, Gating strategy and relative numbers of G-MDSCs ($CD11b^{high}Ly6G^{+}$) and M-MDSCs ($CD11b^{high}Ly6C^{+}$) per $5x10^5$ cells in the BM (top panels) and spleen cells (bottom panels) of the indicated groups.

In SLE patients and lupus-prone mice splenomegaly is observed particularly during active disease. Indeed, splenomegaly was observed in 3 out of 8 diseased NZB/W F1 mice included in this analysis and this was accompanied by increased numbers of splenocytes as compared to young pre-diseased NZB/W F1 animals (**Fig. 7A**). To exclude the possibility that the result of decreased G-MDSCs levels in NZB/W F1 diseased mice was biased by any lymphoproliferative effect, we also analyzed absolute numbers of G-MDSCs per spleen. Although, the absolute numbers of G-MDSCs were significantly increased in the spleen of the lupus mice, their absolute counts did not exceed those of B6 naive mice. In sharp contrast, the spleen size and absolute numbers of G-MDSCs in EAE mice were markedly increased (**Fig. 7B**).

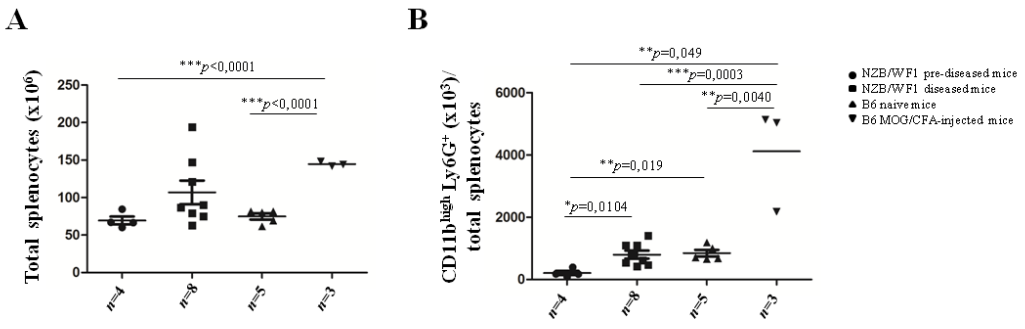


Figure 7. Defective expansion of CD11b^{high}LyG^{+} in the spleen of NZB/W F1 mice. A. Total splenocytes numbers of the indicated groups are shown. **B**. Absolute numbers of CD11b^{high}Ly6G^{+} per spleen. Numbers were extrapolated based on the total number of splenocytes per mouse.

Collectively, these results suggest that the defective MDSC expansion observed in NZB/W F1 mice with established proteinuria is mainly attributed to a selective defect of G-MDSCs.

5.6 Impaired function of CD11b^{high}Ly6-G^{+} G-MDSCs in NZB/W F1 lupus-prone mice

The contrasting result concerning the two subsets expansion in the SLE environment raises further questions about their functionality. To answer this, we checked 7-AAD^{-} CD11c^{-}CD11b^{high}Ly6G^{+}Ly6C^{-} G-MDSCs and 7-AAD^{-}CD11c^{-}CD11b^{high}Ly6G^{-}Ly6C^{+} M-MDSCs suppressive activity on aCD3/aCD28-stimulated CFSE-labeled CD25^{-}CD4^{+} T cells, when the latter were co-cultured with either G-MDSCs or M-MDSCs for 5 days. The results revealed that G-MDSCs promoted the expansion and proliferation of CD4^{+} T cells *in vitro* whereas M-MDSCs slightly suppressed the proliferation of CD4^{+} T cells **(Fig. 8**), supporting the notion that G-MDSC compartment is defective n SLE.

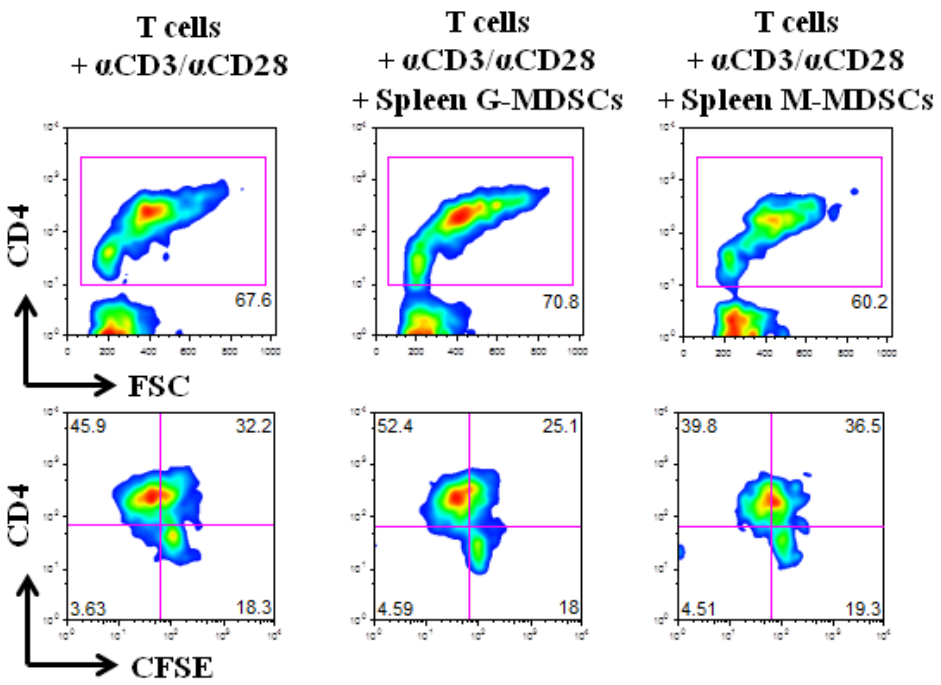


Figure 8. Impaired function of CD11b^{high}Ly6G^{+} G-MDSCs in NZB/W F1 mice. Flow cytometric analysis of CD4^{+} T cells and CFSE dilution of CD4^{+} T cells stimulated by CD3/CD28 beads (beads:T-cells at 1:1 ratio) and co-cultured with the indicated purified cell populations at a 1:1 ratio. Representative results from two independent experiments are depicted.

5.7. CD11b^{high}Ly6-G^{+} G-MDSC impaired expansion is not genetically predisposed in NZB/W F1 mice

One conceivable cause why G-MDSCs are impaired in NZB/W F1 lupus prone mice could be that they are genetically defected and so this is inherited to NZB/W F1 offspring by one or both of the parental strains NZB and NZW mice. Here, it is useful to report that NZB old mice develop spontaneously an autoimmune phenotype (Howie and Helyer 1968) in contrast to the other parental strain (NZW) that are free of disease (Helyer and Howie 1963). Analysis and comparison of G-MDSCs frequency in the spleens of NZB/W F1 mice and their parentals showed that G-MDSCs were

significantly expanded in spleen of NZB but not in NZW old mice (**Fig. 8**). Thus, the impaired expansion of G-MDSCs in NZB/W F1 mice is not a genetic predisposition.

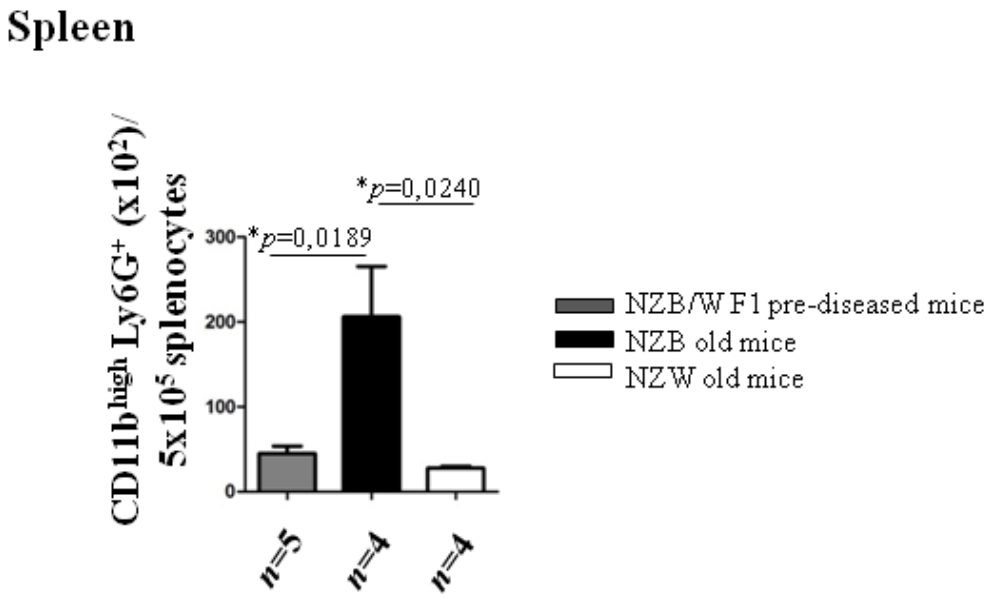


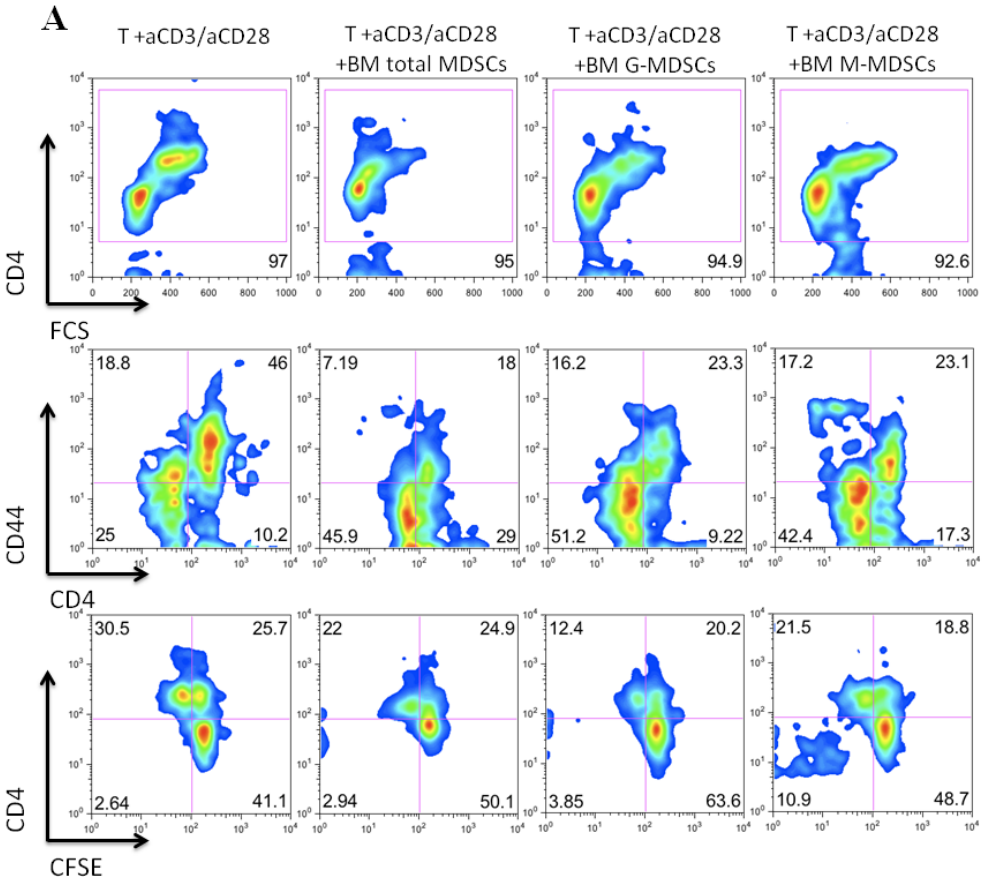
Figure 9. Expansion of CD11bhighLy6G+ G-MDSCs in the spleen of NZB and NZW mice. Relative numbers of $CD11b^{high}Ly6G^{+}$ G-MDSCs/ 5 x 10^5 splenocytes of NZB/W F1 diseased mice and age-matched parental NZB and NZW strains.

Data so far support the notion that the impaired expansion and function of G-MDSCs in NZB/W F1 lupus prone mice could be attributed to lupus microenvironment.

5.8. MDSCs suppressive activity in EAE mice

In a need to strengthen the contradictory to the literature defective function of MDSCs in the inflammatory environment, we sought to exclude any possibility of false results due to any uncontrolled limitations. For this purpose we performed a functional analysis of BM- and spleen- derived total MDSCs and subsets in EAE mice (9 d p.i.),

using the same set-up as with the suppressive assays described above. This experiment confirmed the suppressive activity of both G-MDSCs and M-MDSCs in the BM and spleen of EAE mice (**Fig. 10A and B respectively**), leaving thus no doubts for the validity of the assay.



5.9. Increased spontaneous ETosis in NZB/W F1 mice

Extracellular Trap Formation (ETosis) represents a potential mechanism of cell death of granulocytic cells (Goldmann and Medina 2012). G-MDSCs have granulocytic morphology and are the progenitors of mature granulocytes. Hence, we reasoned that G-MDSCs in NZB/W F1 mice are eliminated through ETosis. On the ground of this hypothesis, we sorted 7-AAD^{-}CD11c^{-}CD11b^{high}Ly6G^{+}Ly6C^{-} G-MDSCs from the BM of NZB/W F1 diseased mice and left them in culture for 4 hours to continue with immunofluoresence in order to assess ET formation. G-MDSCs from B6 naive mice

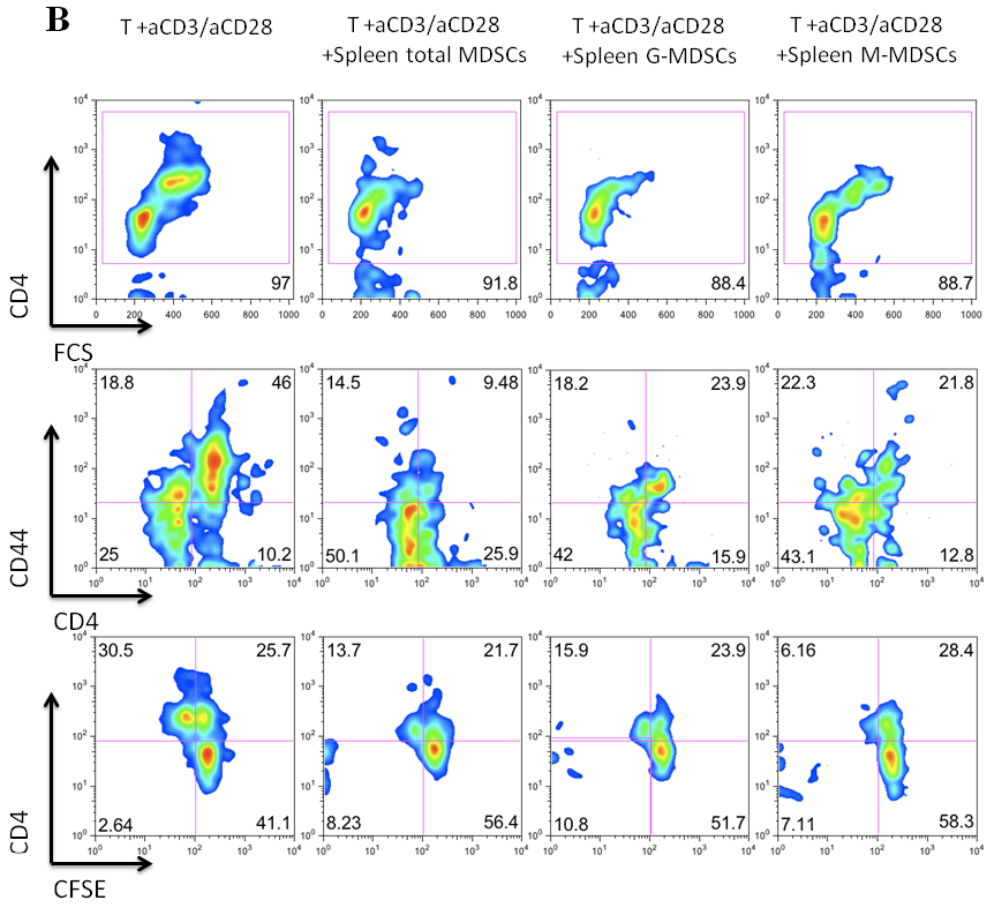


Figure 10. MDSCs suppress T cell responses in EAE mice. Representative flow cytometric analysis of $CD4^+$ T cells, CD44 expression and CFSE dilution of sorted $CD4^+$ T cells stimulated with Dynabeads T-activator CD3/CD28 (beads:T-cells at 1:1 ratio) and co-cultured (5 d) with 4 x 10^4 ***A***, bone marrow- or ***B***, spleen-derived sorted MDSCs (total and subsets). MDSCs and T cells were isolated from B6 CFA-injected mice at d 9 (8-12 wk old), (three independent experiments were performed).

were used as control. PMA induces ETosis and was used as positive control. To this end, NZB/W F1-derived G-MDSCs spontaneously released ETs that appeared as extracellular fiber-like DNA structures in complex with NE (**Fig. 11A**). In total, NZB/W F1 BM G-MDSCs demonstrated enhanced ET release compared to G-MDSCs isolated from B6 control mice (**Fig. 11A and B**). Our data demonstrate that G-MDSCs

are primed to undergo ETosis in lupus mice and this might contribute to their elimination.

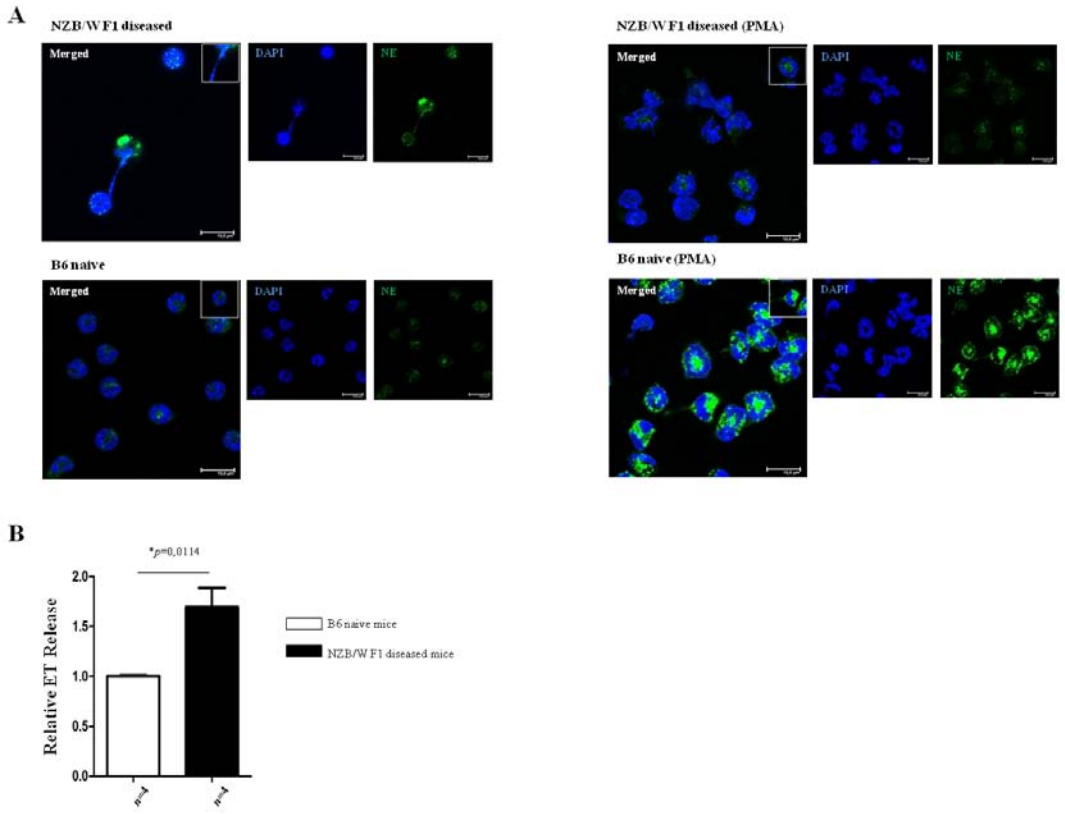


Figure 11. Increased ETosis in lupus G-MDSCs. **A**. Confocal microscopy images showing ET release by sorted $CD11b^{high}Ly6G^{+}$ G-MDSCs (2 x 10^5 cells/ condition) from BM of NZB/W F1 diseased mice and age-matched B6 controls. PMA (100 nM) was used as positive control. DNA stained blue and NE green. Original magnification; x63, zoom; x3, scale bar; 10 μm. **B**. Quantitation of ETs released by pre-diseased and diseased NZB/W F1- versus age-matched B6-derived sorted G-MDSCs. Results in **A** and **B** are representative of four independent experiments.

5.10. NZB/W F1 lupus microenvironment induces extracellular trap (ET) formation by G-MDSCs

Our data so far reveal a defect in the G-MDSC compartment in SLE and raise the possibility of an intrinsic effect driven by the inflammatory environment of lupus. Along these lines, we further examined whether the inflammatory environment of lupus could drive ET formation by G-MDSCs. Immature $Ly6G^+$ granulocytic cells were isolated from BM of B6 naive mice and treated with 2% serum isolated from NZB/W F1 pre- or diseased or age-matched B6 mice in order to assess ETosis. $Ly6G^+$ cells were isolated on a percoll gradient (>80% purity). Of note, NZB/W F1 serum from mice with proteinuria potently induced ET release by $Ly6G^+$ cells in contrast to serum from NZB/W F1 pre-diseased mice that induced ETs to a lesser extent. The control serum from B6 naive mice was unable to drive ET formation. Lupus serum-induced ETs were characterized by the presence of NE and MPO co-localized with DNA (**Fig. 12A and B**). These results support that the inflammatory milieu of lupus mediates elimination of G-MDSCs through ETosis.

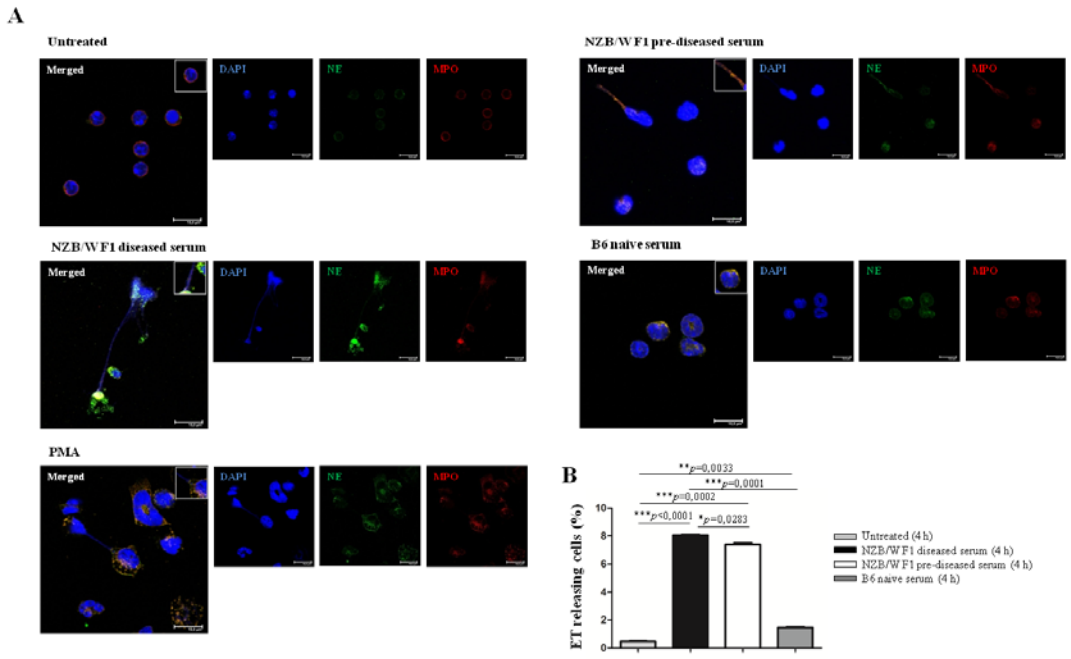


Figure 12. Lupus serum drives ETosis in $Ly6G^+$ cells. A. Confocal microscopy analysis for ET formation by naive B6-derived BM-isolated $Ly6G^+$ cells (2 x 10^5 cells/condition) treated with

2% NZB/W F1 pre-diseased and diseased serum, B6 serum or PMA (100 nM). Untreated B6-derived BM-isolated Ly6G$^+$ cells were used as negative control. DNA stained blue, NE green and MPO red. Original magnification; x63, zoom; x3, scale bar; 10μm. Representative results from fifteen independent experiments. **B**. Quantitation of ETs released by untreated and indicated serum-treated BM-isolated Ly6G$^+$ cells.

To preclude the possibility that other types of cell death are induced by NZB/W F1 serum in Ly6G$^+$ cells we performed a 7-AAD/ Annexin V apoptosis assay. Ly6G$^+$ cells were isolated from B6 naive mice and treated with NZB/W F1 diseased-derived serum in vitro for 4 hours. FBS and serum isolated from B6 mice, naive or EAE, were used as controls. Our results revealed no significant differences in the frequency of necrotic (7-AAD$^+$Annexin V$^{-/+}$) and apoptotic (7-AAD$^-$Annexin V$^+$) Ly6G$^+$ granulocytic cells in the presence of sera used (**Fig. 13**). As such, we conclude that Ly6G$^+$ die through ETosis in the inflammatory environment of lupus.

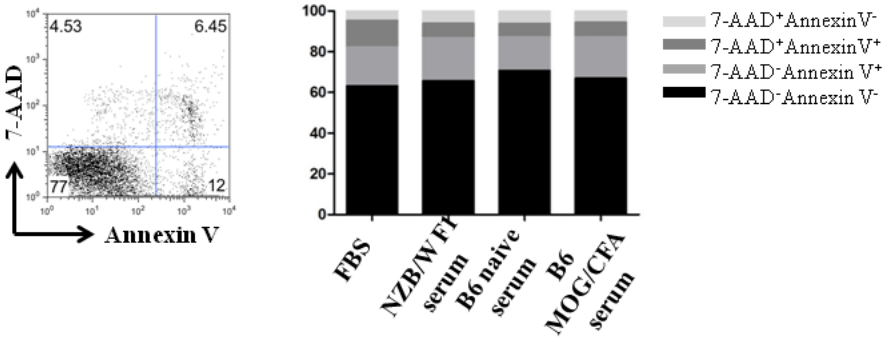


Figure 13. Cell viability assay of Ly6G$^+$ cells in the presence of NZB/W F1–derived serum. Annexin V and 7-AAD staining on B6-derived BM-isolated Ly6G$^+$ cells treated as indicated. Numbers denote frequency. Mean percentages of viable (7-AAD$^-$AnnexinV$^-$), apoptotic (7-AAD$^-$Annexin V$^+$) and necrotic (7-AAD$^+$Annexin V$^{-/+}$) cells are shown. Data are representative of four independent experiments.

5.11. Lupus milieu reverses the suppressive properties of G-MDSCs from NZB/W F1 male mice

Male NZB/W F1 mice do not develop spontaneous autoimmune disease and so their cells, including MDSCs, are not exposed to inflammatory stimuli similar to those of lupus milieu. Therefore NZB/W F1 male mice constitute a very good source of MDSCs that are certainly not primed before isolation and carry similar genetic information as MDSCs from females. Consequently, we questioned what is the suppressive capacity of male-derived MDSCs and, importantly, what is the effect of female NZB/W F1 diseased-derived serum on it. For this, we sorted G-MDSCs from the spleen of male NZB/W F1 mice (as described above), pretreated them for 4 h *in vitro* with lupus serum isolated from female NZB/W F1 mice, then had them washed and left them in culture with syngenic aCD3/aCD28-stimulated CFSE-labeled $CD25^{-}CD4^{+}$ T cells. Untreated G-MDSCs were used as control. Interestingly, although male G-MDSCs suppressed the activation and proliferation of $CD4^{+}$ T cell *in vitro*, pre-treatment with lupus serum, resulted in loss of their suppressive function and this was accompanied by increased ET formation. (**Fig. 14**). Collectively, our data indicate that lupus microenvironment alters the suppressive activity of MDSCs.

5.12 Lupus inflammatory milieu induces ETosis in Ly6G+ granulocytes through generation of ROS

5.12.a. Lupus inflammatory milieu induces ROS production

Induction of ETs by neutrophils has been shown to be dependent on activation of autophagy pathway and NADPH oxidase-mediated oxidative burst (Remijsen, Vanden Berghe et al. 2011). Initially we designed a Western blot analysis on protein lysates isolated from $Ly6G^{+}$ cells treated with NZB/W F1 diseased-derived serum or B6 naive serum as a control. We checked for the lipidation of LC3, an essential procedure for

autophagy (Kabeya, Mizushima et al. 2000) and the degradation of p62, the

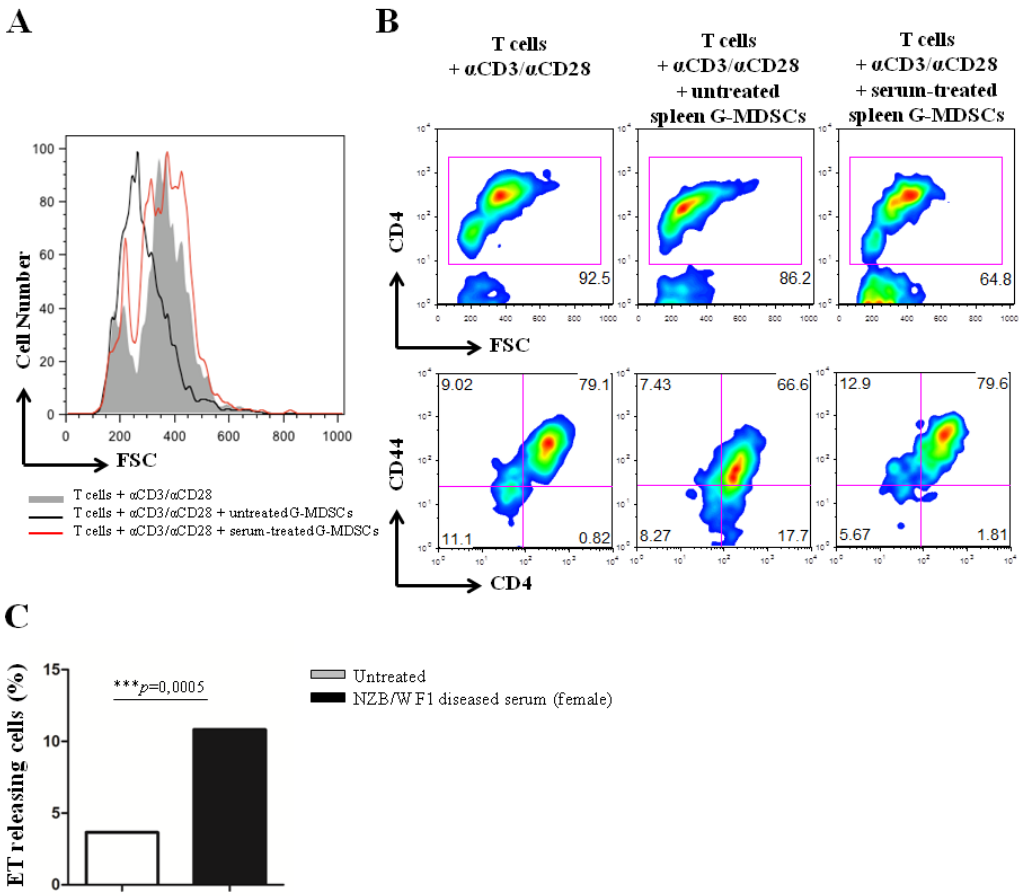


Figure 14. Lupus milieu reverses the suppressive properties of G-MDSCs from NZB/W F1 male mice. Flow cytometric analysis of **A**, Forward Scatter (FSC) and **B**, CD44 expression on male NZB/W F1-derived sorted $CD4^+$ T cells stimulated by CD3/CD28 beads (beads:T-cells at 1:1 ratio) and co-cultured with syngeneic splenic sorted G-MDSCs untreated or treated with 2% NZB/W F1-derived lupus serum at a 1:1 ratio. Representative results from two independent experiments are shown. **C.** Quantitation of ETs is shown. Data are representative of three independent experiments.

autophagic adaptor protein that links ubiquitinated substrates to autophagy pathway, and facilitates the completion of the autophagic pathway (Ponpuak, Davis et al. 2010). Neuro2A cell line lysates were used as positive controls for LC3 lipidation and

autophagy completion. Our results show that treatment of $Ly6G^+$ cells with either diseased NZB/W F1 or naive B6 serum did not result in significant differences regarding the lipidation of LC3. In addition, we did not observe increased degradation of p62 (**Fig. 15A**). These results suggest that the autophagic pathway might not be implicated in ET formation by G-MDSCs in lupus.

Next, we assessed whether ROS production by $Ly6G^+$ cells is triggered by lupus serum. For this, we treated $Ly6G^+$ cells with lupus serum and appropriate control sera (FBS and B6 naive derived-serum) and measured total ROS production by FACS analysis with the use of DCF that detects cellular reactive oxygen species. PMA induces oxidative stress that results excessive ROS production and so it was used as positive control. As shown in **Fig. 15B**, serum from NZB/W F1 diseased mice promoted ROS production by $Ly6G^+$ cells compared to serum from B6 naive mice.

In order to determine the specificity of the observed effect we repeated the assay with adding NAC, a compound with free radical scavenging properties. The addition of NAC reduced ROS expression in lupus serum-treated (**Fig. 15C**) and PMA-treated (data not shown) $Ly6G^+$ cells suggesting that the inflammatory environment of lupus specifically promotes ROS production in $Ly6G^+$ granulocytes.

BM-isolated $Ly6G^+$ cells were triggered with (**B**) 2% FBS, NZB/W F1 diseased or B6 naive serum and PMA (eight independent experiments) or (**C**) with 2% FBS or NZB/W F1 diseased serum together with 20 mM NAC (four independent experiments) for 1 h in the presence of 5 μM DCF. Total ROS release was measured with flow cytometry.

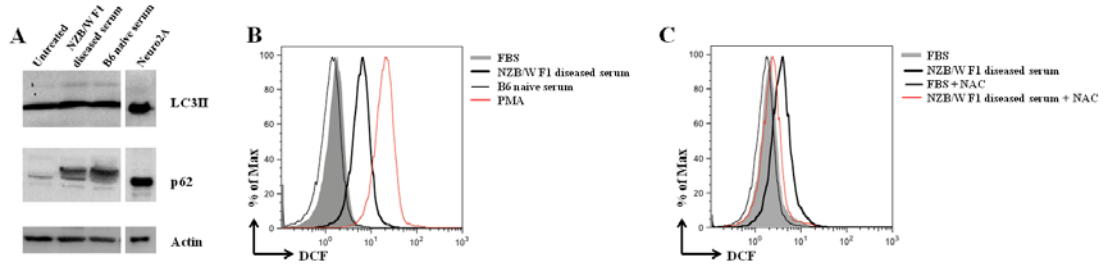


Figure 15. **The lupus milieu triggers ROS production in Ly6G$^+$ cells.** **A**. Western blot analysis of LC3 and p62 expression by B6 naive-derived BM-isolated Ly6G$^+$ cells untreated and treated with 2% NZB/W F1 diseased or B6 naive serum for 2h. **B** and **C**. B6 naive-derived

5.12.b. ROS dependent generation of ETs by LY6G$^+$ cells

Due to the lupus serum property to induce ROS production by Ly6G$^+$ cells, an important issue arose; whether serum-induced ROS production by the aforementioned cells mediates ET release. To answer this we treated Ly6G$^+$ cells isolated from B6 naive mice with NZB/W F1 serum from diseased mice in the presence of NAC, to have ROS scavenged, and assessed ETosis, according to the commonly used protocol. FBS-treated cells were used as control. Importantly, NAC significantly reduced ET formation by lupus serum-treated B6 naive-derived BM Ly6G$^+$ cells (**Fig. 16**) providing additional evidence for the importance of ROS generation in ET formation by Ly6G$^+$ cells.

5.13. Lupus inflammatory milieu induces mitochondria-derived ROS production in Ly6G$^+$ granulocytes.

Various organelles within the cell can generate ROS, including mitochondria (mt), the endoplasmic reticulum (ER) and peroxisomes (as part of their role in metabolizing long-chain fatty acids). In addition, various enzymes, including oxidases, as for

example NADPH, and oxygenases, generate ROS as part of their enzymatic reaction cycles (Holmstrom and Finkel 2014). Thinking about the various sources of ROS, we

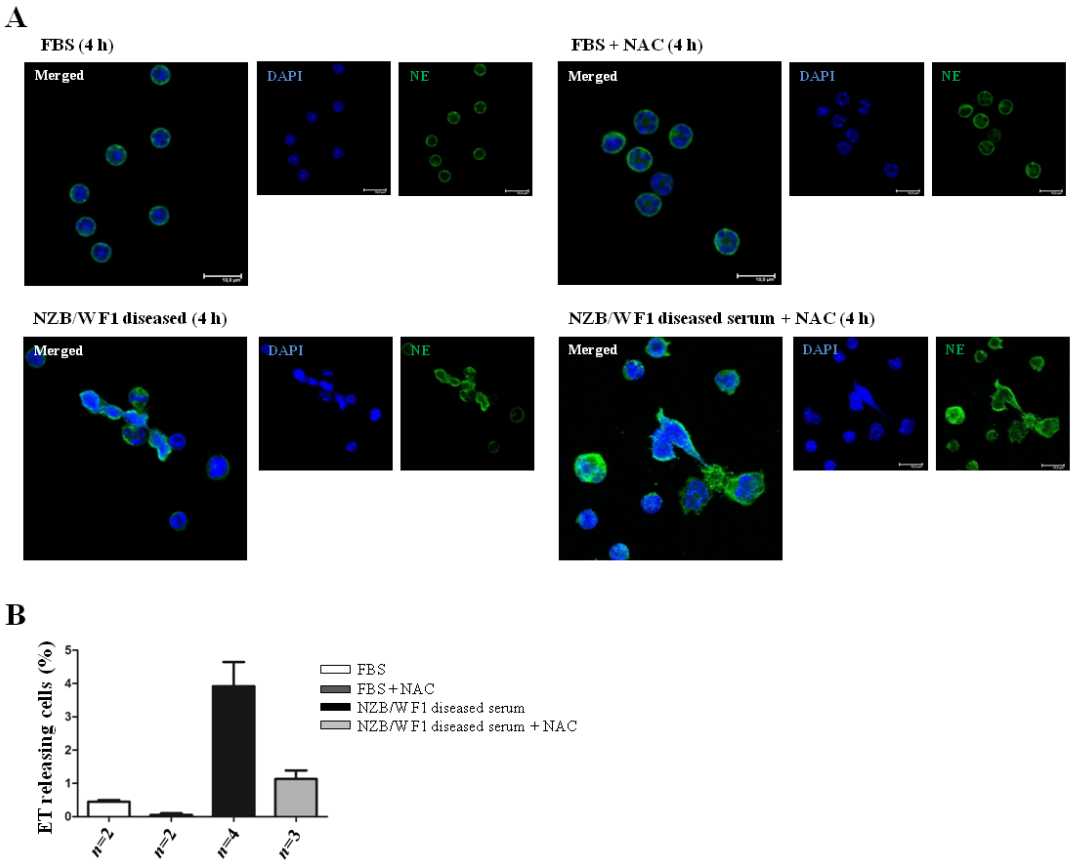


Figure 16. ROS-dependent generation of ETs by Ly6G$^+$ cells, in NZB/W F1 mice. A. Confocal microscopy images of ET release from B6 naive-derived BM-isolated Ly6G$^+$ granulocytes treated with 2% FBS or NZB/W F1 diseased mouse serum, in the presence and absence of NAC (20 mM). DAPI and NE are shown. Original magnification; x63, zoom; x3, scale bar; 10 μm. **B.** Quantitation of ETs released by Ly6G$^+$ cells treated with diseased NZB/W F1 serum in the presence or absence of NAC.

sought to examine the source of ROS that are produced by lupus serum-treated Ly6G$^+$ cells that contributes to ET formation. At first we used the DPI reagent that specifically scavenges NADPH-produced ROS. Inhibition of NADPH oxidase activity by DPI (20 μM) slightly attenuated ROS production in NZB/W F1 serum-treated B6 naive-derived BM Ly6G$^+$ cells (data not shown) suggesting that ROS production in B6-

derived BM Ly6G$^+$ cells might not require NADPH activity. Thus, we asked whether mitochondria contribute to ROS generation in Ly6G$^+$ cells exposed to lupus environment. To this end, treatment of Ly6G$^+$ cells with NZB/W F1 serum from diseased mice in the presence of MitoSox, that detects mt-produced ROS, revealed a marked increase of mtROS production (**Fig. 17**). As positive control, cells were stimulated with 500 μM H_2O_2 (data not shown). Collectively, these data suggest a possible role of mitochondria-derived ROS in ET formation by Ly6G$^+$ cells.

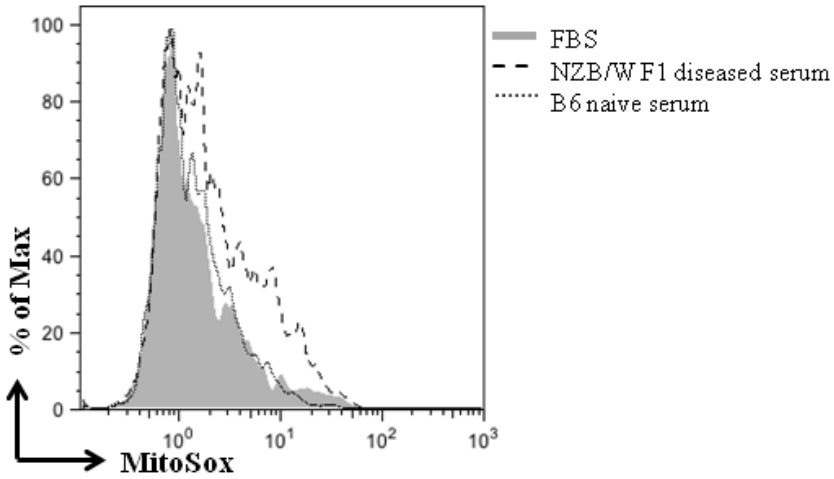


Figure 17. **Lupus serum-induced mitochondrial ROS production in Ly6G+ cells.** BM-isolated Ly6G$^+$ cells from B6 naive mice were treated with serum isolated from NZB/W F1 diseased mice or B6 naive control serum and mtROS production was measured with MitoSox (5 μM). Representative of two independent experiments are shown.

5.14. IFN-α, IFN-γ and IL-6 induce ROS generation by Ly6G$^+$ cells

To elucidate molecules that could drive ET formation in Ly6G$^+$ cells, we focused on cytokines that are enriched in lupus inflammatory milieu and are linked to SLE pathogenesis, such as IFN-α, IFN-γ and IL-6 (Ohl and Tenbrock 2011). A control experiment with FACS analysis confirmed first that all three cytokine receptors were expressed by NZB/W F1 Ly6G$^+$ cells (**Fig. 18A**) and so these cells could be potently triggered by the respective cytokines. Then, we triggered B6 naive-derived Ly6G$^+$

cells with autologous serum (control) and lupus serum to assess whether the inflammatory environment of lupus can promote IFN-αR, IFN-γR and IL-6R receptors expression. The FACS analysis revealed that treatment of B6-derived BM Ly6G$^+$ cells with NZB/W F1 serum up-regulated only IFN-γR expression but not IFN-αR or IL-6R expression (**Fig. 18B**). Furthermore, treatment of B6 naive-derived BM Ly6G$^+$ cells with rIFN-α, rIFN-γ or rIL-6 resulted in increased ROS production compared to untreated cells (**Fig. 18C**).

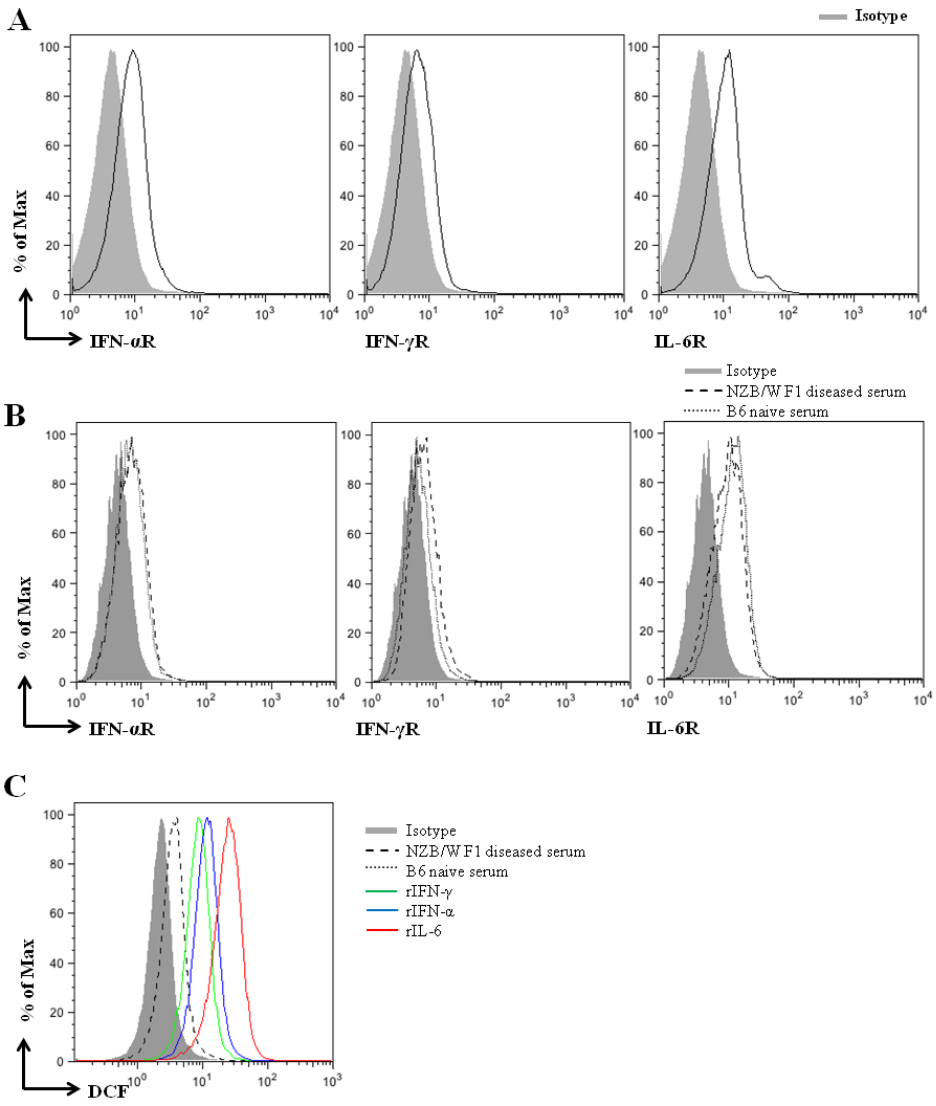


Figure 18. rIFN-α, rIFN-γ and rIL-6 promotes ROS generation in Ly6G$^+$ cells. **A** and **B**. Expression levels of IFN-αR, IFN-γR and IL-6R by (**A**) diseased NZB/W F1-derived BM G-

MDSCs or (**B**) naive B6-derived BM Ly6G$^+$ cells treated with 2% B6 or NZB/W F1 diseased serum for 4 h *in vitro.* Representative data of two independent experiments (two replicates each). **C***.* Naive B6–derived BM-isolated Ly6G$^+$ cells (2 x 10^5 cells/condition) were treated with NZB/W F1 diseased or control serum, rIFN-α (10^4 U/ml), rIFN-γ (20 ng/ml) or rIL-6 (30 ng/ml) cytokine for 1 h *in vitro.* ROS was detected upon addition of 5 μM DCF by FACS. Data shown represent three independent experiments of two replicates each.

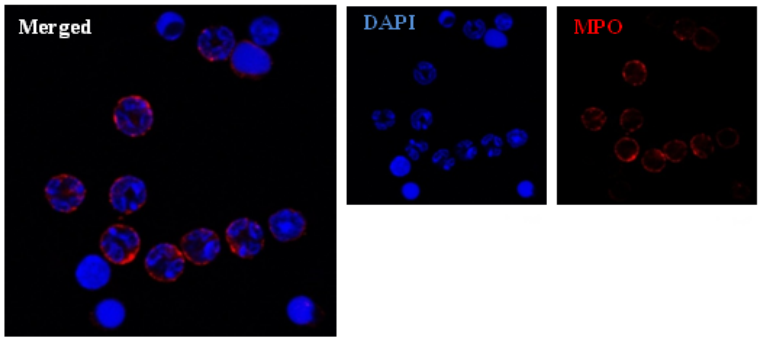
5.15. IFN-α, IFN-γ and IL-6 promote ETosis by Ly6G$^+$ cells

The contribution of the IFN-α, IFN-γ and IL-6 on serum-mediated ETosis, was determined by immunofluoresence of B6-derived BM Ly6G$^+$ cells treated individually with the rIFN-α, rIFN-γ or rIL-6. This experiment demonstrated that treatment of these Ly6G$^+$ cells with each one of the three cytokines resulted in the formation of ETs that stained positively for MPO (**Fig. 19**). Taken together, our data support the notion that IFN-α, IFN-γ as well as IL-6 mediate ET release by Ly6G$^+$ granulocytes, possibly through the promotion of ROS production.

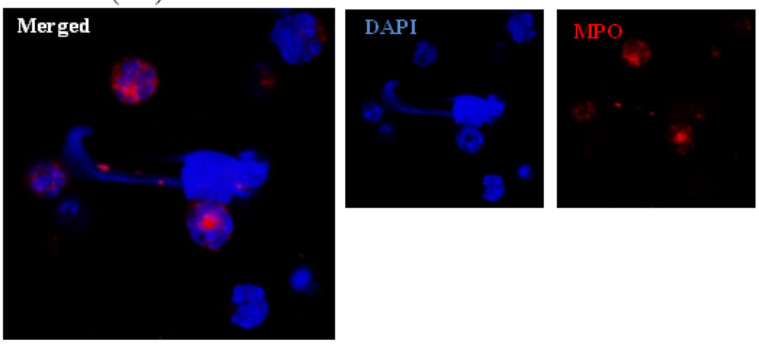
5.16. Lupus G-MDSCs are immature myelocytes, distinct from mature neutrophils

Due to morphological similarities that G-MDSCs and neutrophils share it often appears necessary to characterize further these cells. Under this scope, we chose to compare the phenotype of G-MDSCs and neutrophils isolated from the spleen of B6 mice immunized with CFA and additionally check these two populations functionality in regards with their effect on T cells responses. Figure 20A shows the gating strategy that was followed to sort the two populations. G-MDSCs were sorted as for all experiments till now and according to the well-established gating strategy described originally by Gabrilovich and colleagues in tumor immunology field (Gabrilovich and Nagaraj 2009). “Regular” neutrophils were characterized as CD11b$^-$Gr1$^+$, a population that also expanded in the spleen of B6 mice upon CFA immunization.

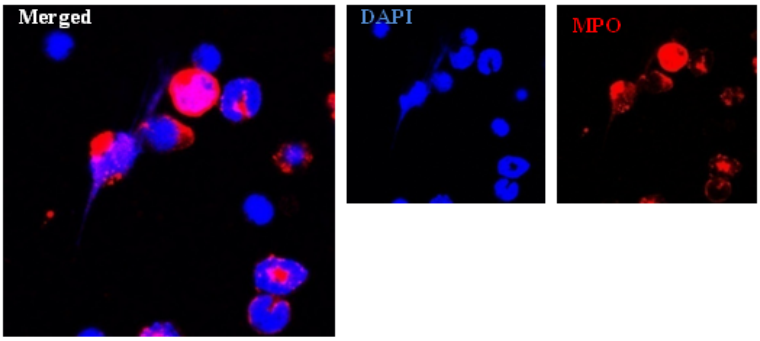
Untreated (4 h)



rIFN-α (4 h)



rIFN-γ (4 h)



rIL-6 (4 h)

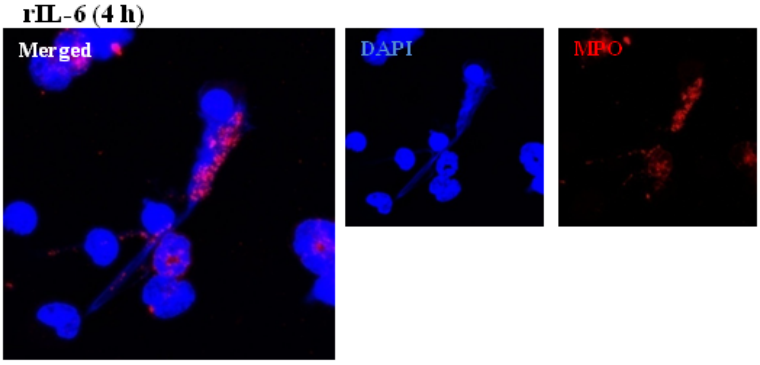


Figure 19. **rIFN-α, rIFN-γ and rIL-6 promote ETosis in Ly6G$^+$ cells.** Confocal microscopy images representative of four independent experiments (2 replicates each) that show ET release from naive B6-derived BM-isolated Ly6G$^+$ cells (2 x 10^5 cells/condition) treated with rIFN-α (10^4 U/ml) or rIFN-γ (20 ng/ml) or rIL-6 (30 ng/ml). DNA is stained blue and MPO red. Original magnification; x63, zoom; x3, scale bar; 10 μm.

The phenotypic characterization of the cells relied on nucleus morphology observation under microscopy. Upon staining with DAPI, we observed that cells belonging to the so-called G-MDSCs compartment have ring-shaped nucleus; conversely, cells that belong to the CD11b$^-$Gr-1$^+$ population had hyper-segmented nucleus indicative of terminally differentiated, mature granulocytes (**Fig. 20B**).

Apart from the nuclear morphology, we performed a functional assay to compare the two populations. To this end, we sorted the abovementioned cell populations and assessed their capacity to suppress T cell responses *in vitro*. We used the same design as described above for the suppressive assays. As shown below, only G-MDSCs (CD11b^{high}Ly6G$^+$ cells) from CFA-immunized animals were able to suppress T cell activation and proliferation, whereas CD11b$^-$Gr1$^+$ neutrophils did not (**Fig. 20C)**.

Collectively, although there is still lack of consensus regarding the distinction between G-MDSCs and mature neutrophils, we believe that based on the morphology as well as on the *in vitro* suppressive activity, the two subsets belong to different maturation and/or developmental stages and thus, the functional properties of these subsets should always be adequately addressed.

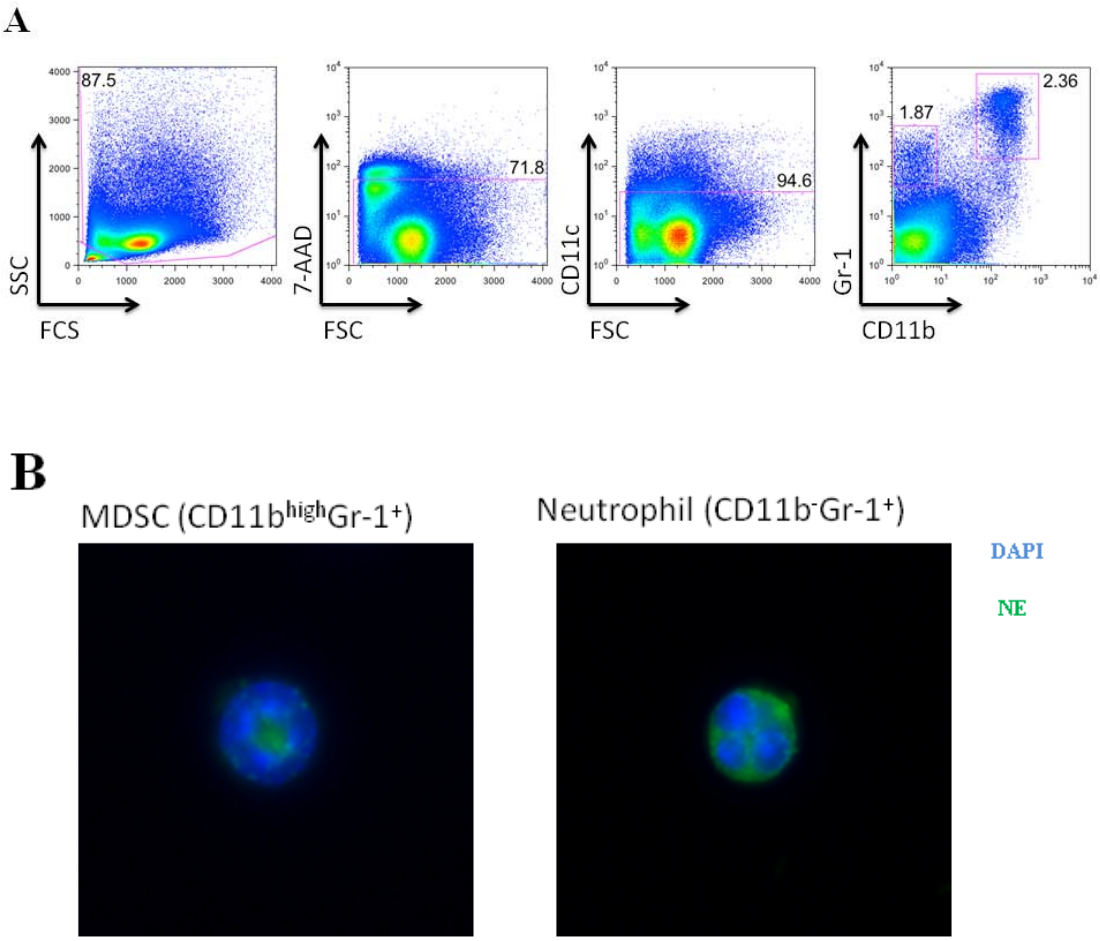


Figure 20. Distinct phenotypic and functional properties between G-MDSCs and regular neutrophils. A. Gating strategy for MDSCs ($CD11b^{high}Gr\text{-}1^{+}$) and "normal" neutrophils ($CD11b^{-}Gr\text{-}1^{+}$) cell sorting from spleen of B6 CFA-injected mice (d9). **B**. Microscopy images showing the nucleus morphology and neutrophil elastase staining in MDSCs ($CD11b^{high}Gr\text{-}1^{+}$) and neutrophils ($CD11b^{-}Gr\text{-}1^{+}$) sorted from the spleen of B6 CFA-injected mice (d9).

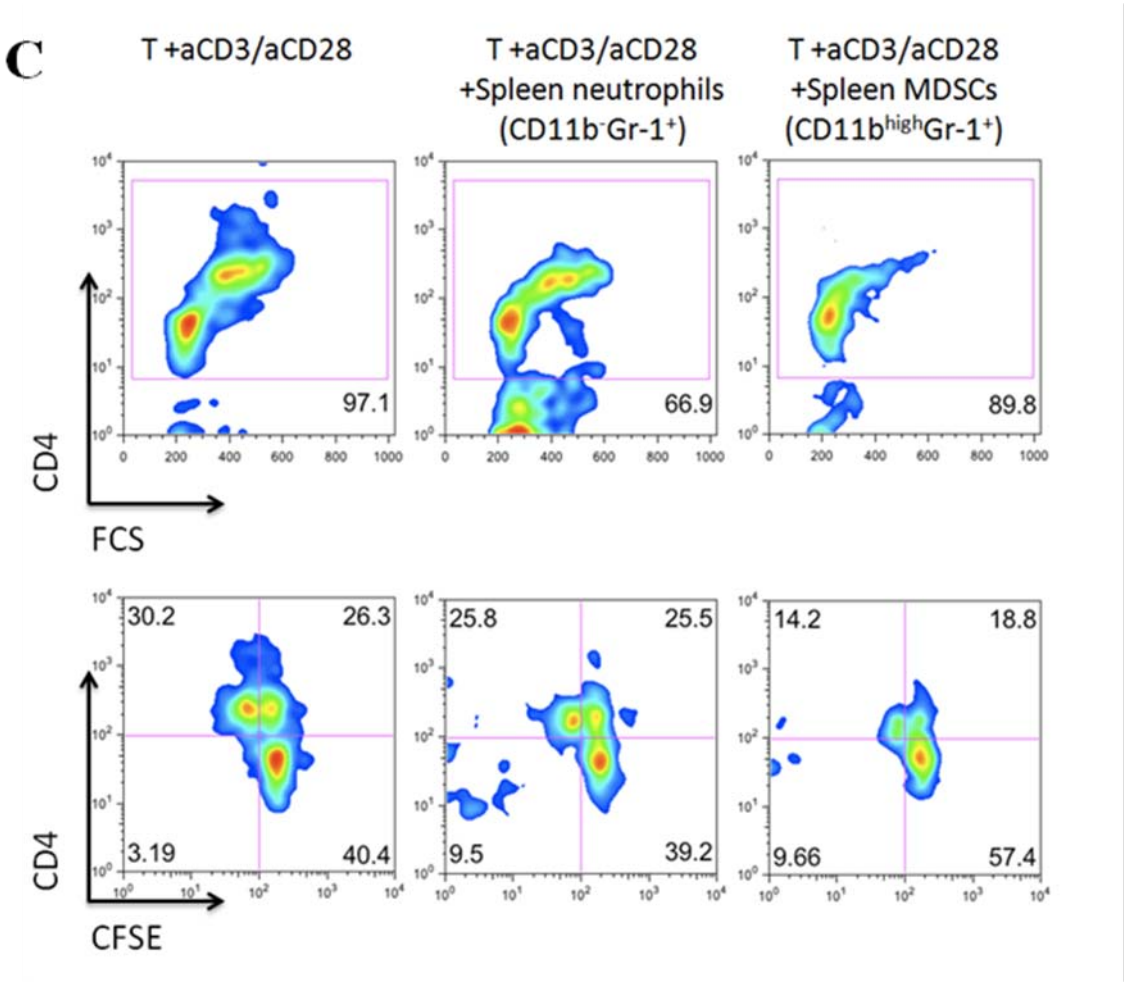


Figure 20 (continued). Distinct phenotypic and functional properties between G-MDSCs and regular neutrophils. C. Representative flow cytometric analysis of $CD4^+$ T cells and CFSE dilution of sorted $CD4^+$ T cells stimulated with Dynabeads T-activator CD3/CD28 (beads:T cells at 1:1 ratio) and co-cultured (5 d) with 4 x 10^4 spleen-derived sorted $CD11b^-Gr\text{-}1^+$ neutrophils and $CD11b^{high}Gr\text{-}1^+$ MDSCs (neutrophils/MDSCs:T cells at 1:1 ratio). All cell populations were isolated from B6 CFA-injected mice at d 9 (8-12 wk old). Results are representative of three independent experiments.

6. DISCUSSION

The loss of self tolerance determinates the development of autoimmune responses. The continuous immune stimulation with self antigens in the presence of a deregulated immune system inevitably leads to the maintenance of autoimmune responses and progressive establishment of chronic inflammation. To date, various cell subsets have been proposed to possess an immunosuppressive role in autoimmune responses including Tregs, regulatory B cells, type II monocytes and MDSCs. However, the function of regulatory networks in the field of autoimmnune diseases is not completely understood. Understanding of the regulatory mechanisms of immune responses prior to chronic inflammation establishment will allow development of new, more efficient therapeutic approaches. In the present study, we provide evidence for an impaired expansion of the G-MDSC regulatory compartment of the innate immune system under the lupus inflammatory environment. Lupus serum induces the formation of ETs by G-MDSCs thus leading to their elimination in a ROS-dependent manner. Together, these data demonstrate the defective operation of a regulatory cell subset that might contribute to SLE pathogenesis.

MDSCs and their regulatory role in tolerance maintenance in autoimmunity have recently gained interest. MDSCs are known to expand upon inflammation, exert the bone marrow where they reside and regulate immune responses in the periphery by suppressing T cell proliferation and activation. Such a role has been already assigned to MDSCs in rheumatoid arthritis (Fujii, Ashihara et al. 2013; Kurko, Vida et al. 2014), multiple sclerosis (Zhu, Bando et al. 2007; Ioannou, Alissafi et al. 2012), type I diabetes (Yin, Ma et al. 2010; Whitfield-Larry, Felton et al. 2014) and inflammatory bowel disease (Guan, Moreno et al. 2013; Xi, Li et al. 2015). However, the role of MDSCs in SLE where inflammation often remains unresolved had not been

addressed. Moreover, previous reports suggested a significant decrease in circulating Tregs and impaired function (Lee, Wang et al. 2006; Suen and Chiang 2012), although their role remains controversial (Azab, Bassyouni et al. 2008). Hence, thorough investigation of MDSCs regulatory role in SLE appears necessary. Surprisingly enough, analysis of total MDSC frequency in the bone marrow and spleen of NZB/W F1 diseased mice revealed that $CD11b^{high}Gr\text{-}1^{+}$ cells are eliminated during active disease, despite the ongoing inflammation. There are many possible explanations why these cells could be eliminated. We excluded the possibility that the NZB/W F1 mice BM is incompetent, since immunization of these mice with CFA was accompanied by MDSC expansion. Furthermore, the low levels of MDSCs in the spleen of diseased mice could not be assigned to MDSC migration to the inflammatory site, as FACS analysis in the kidneys and rLNs defeated this scenario. The possibility of a genetic predisposition was rejected as well after analysis of MDSCs expansion in the parental strain NZB. NZB old mice that spontaneously develop an autoimmune phenotype (Howie and Helyer 1968) had elevated levels of MDSCs in the bone marrow and the spleen. Taking into account these findings we formulated the hypothesis that MDSCs are eliminated in lupus mice due to the inflammatory environment of lupus.

Before examining the hypothesis of the lupus inflammatory environment effect, we looked at the frequency two MDSCs subsets separately. As mentioned earlier MDSCs are a heterogeneous population of two distinct subsets, namely the G-MDSCs and M-MDSCs. Evidence indicates that these two subpopulations may have different functions in cancer and infectious and autoimmune diseases (Dietlin, Hofman et al. 2007; Zhu, Bando et al. 2007; Movahedi, Guilliams et al. 2008; Ioannou, Alissafi et al. 2012). Our analysis showed that the defective MDSC expansion observed in NZB/W F1 mice with established proteinuria is mainly attributed to a selective defect of G-

MDSCs. Probably the reason for this selective elimination is that the cytokines, autoantibodies and/or immune complexes present in SLE may alter the bone marrow niche and/or promote the release of immature monocytic MDSCs; yet M-MDSCs levels were significantly increased only in the periphery of lupus mice. Alternatively, the eliminated levels of MDSCs in lupus may be to a trend of an orientated, rapid differentiation of immature myeloid cells into effector cells (macrophages, dendritic cells and granulocytes/neutrophils) in response to autoimmune reactivity; this is a hypothesis that is under investigation. An altered chemotactic response could also be a claim, but in view of this aspect we would expect to have accumulation of MDSCs in the bone marrow, which is not the case. Considering the fact that G-MDSCs are the progenitors of neutrophils, that are known to exert increased NETosis which correlates with SLE disease pathogenesis we formulated the hypothesis that G-MDSCs are eliminated in the inflammatory environment of lupus through extracellular trap formation.

In the periphery of active SLE patients neutropenia due to increased apoptosis of neutrophils has been reported (Ren, Tang et al. 2003; Midgley, McLaren et al. 2009). Bone marrow from lupus patients has reduced granulocyte-macrophage colony forming units (Papadaki, Boumpas et al. 2001; Wahren-Herlenius and Dorner 2013) and decreased frequency of newly generated neutrophils (Orr, Taylor et al. 2005). It is true that the discrimination of G-MDSCs and neutrophils still lack a consensus and identification of better markers is mandatory. Nonetheless, these two cell populations have different phenotype and functionality in the grounds of immune suppression. We show in our study that $CD11b^{high}Gr\text{-}1^{high}$ G-MDSCs have a ring-shaped nucleus, typical of granulocytic cells at an early differentiation state (Pillay, Tak et al. 2013) in contrast to “regular” neutrophils ($CD11b^{-}Gr\text{-}1^{+}$) which have a hypersegmented nucleus, a key characteristic of terminally differentiated neutrophils (Pillay, Tak et al.

2013). Moreover, these two subsets differed functionally; G-MDSCs could suppress T cell responses, while neutrophils could not. Ergo, we are confident that the G-MDSC compartment that is studied in this project is different than mature neutrophils, the role of which in SLE has been already assessed in previous studies.

Neutrophil extracellular trap formation (NETosis) is exacerbated in SLE patients and is involved in disease pathogenesis (Villanueva, Yalavarthi et al. 2011; Dorner 2012). The effect of this phenomenon is pleiotropic. It has been shown that SLE NETs trigger pDC activation to produce type I IFN, a key cytokine in lupus pathogenesis (Garcia-Romo, Caielli et al. 2011). Lande *et al*. have shown that neutrophils activate pDCs by releasing NETs containing self-DNA-peptide complexes (Lande, Ganguly et al. 2011). Importantly, NET releasing neutrophils induce endothelial dysfunction in SLE through the activation of matrix metalloproteinase-2 (Carmona-Rivera, Zhao et al. 2015). All the studies above deliberate extracellular trap formation by mature neutrophils, according to the isolation techniques they use. Our study is the first to show that lupus G-MDSCs, the progenitors of mature neutrophils, are prone to spontaneously develop extracellular traps. Of interest, NETosis has been well investigated in SLE, in a newly identified compartment of granulocytes in SLE patients, named as low density granulocytes (LDGs), (Denny, Yalavarthi et al. 2010; Villanueva, Yalavarthi et al. 2011; Carmona-Rivera, Zhao et al. 2015). LDGs have been characterized as a distinct population of mature neutrophils, however, they must not be G-MDSCs since microscopy observation of the LDG nuclei revealed a mixed population of granulocytes at three differentiation states (round, segmented and banded nuclei), (Denny, Yalavarthi et al. 2010). As such, our observation of increased spontaneous ETosis by G-MDSCs in SLE is novel and offers new insights in the contribution of the innate immune system in the pathogenesis of lupus.

As cited earlier in this context, aberrant NETosis is related to unfavorable effects on immune system homeostasis and contributes to the immunopathogenesis of SLE. Apart from the dramatic effects that ETosis of the G-MDSC compartment has in the perpetuation of adaptive immune responses, there is one extra risk in this case; the acquired loss of a regulatory compartment early in the development of the disease which in turn deregulates immunosuppressive mechanisms and autoimmune responses are burdened. This early on the disease development effect is supported by the fact that ETosis occurs in G-MDSCs even in NZB/W F1 pre-diseased mice, before inflammation is established in the kidneys and proteinuria is manifesting. In parallel, increased ETosis by G-MDSCs, in concert with low serum DNase 1 activity (Gajic-Veljic, Bonaci-Nikolic et al. 2015) and impaired phagocytosis of cell debris (Herrmann, Voll et al. 1998) in the periphery of SLE patients may yield novel autoantigens that persist in the periphery and consequently lead to the production of autoantibodies. In support, Hakkim *et al.* have shown that impaired degradation of NETs due to the presence of DNase1 inhibitors and the restricted access of DNase1 to the anti-NET antibodies-covered NETs correlated with lupus nephritis in SLE patients (Hakkim, Furnrohr et al. 2010). Moreover, we cannot exclude the possibility that the exposure of immature lymphocytes to self antigens in the bone marrow during lymphocyte maturation might have detrimental effects in the survival of self-reactive lymphocytes, as tolerogenic mechanisms greatly depend on the concentration of a self antigen in the bone marrow. G-MDSCs are progenitors of mature granulocytes, that are responsible for eliminating microbes. Correspondingly, increased elimination of G-MDSCs through ETosis might also have an effect in the abundance of effector cells, which could possibly compose an extra risk factor for the increased susceptibility of SLE patients to opportunistic infections. Conclusively, the effects of a deregulated pathway of the immune system can be boundless; therefore it would have been more

gainful to focus on the mechanisms that are responsible for ETosis in the G-MDSC compartment. Delineating the causative mechanisms will provide opportunities for the development of new, targeted and more efficient therapies.

Elimination of G-MDSCs was specific for NZB/W F1 lupus-prone mice. Taking for granted that MDSCs receive signals from their environment, including inflammatory cytokines, that determine their expansion and function (Gabrilovich and Nagaraj 2009; Condamine and Gabrilovich 2011), we hypothesized that the increased ETosis observed in this subset is driven by the inflammatory milieu of lupus. Indeed, thorough examination of this hypothesis revealed that the serum isolated from mice with established proteinuria was enough to promote ETosis in cells that were not otherwise prone to release ETs. Among other common constituents of SLE serum, patients have elevated IFN-α, IFN-γ and IL-6 levels. In this study we show that all these three cytokines can drive extracellular trap formation by G-MDSCs. Other surveys also report the role of IFN-α and IFN-γ as inducers of NETosis (Knight and Kaplan 2012). However Martinelly *et al.* have shown that IFN-α and IFN-γ promote extracellular trap formation only in mature neutrophils and not in immature myeloblasts, which generally respond to both cytokines with lower efficacy (Martinelli, Urosevic et al. 2004). The role of IL-6 in the initiation of NETosis remains unanswered. Of note, our data reveal an induced IFN-γR expression on $Ly6G^+$ cells treated with lupus serum, that possibly correlates with triggering ET release. Counting in that these three cytokines alter MDSCs status through different signaling pathways, it appears necessary to investigate further their role in promoting G-MDSCs ET release, ideally by looking at molecules downstream their receptors. Despite the clear role of IFN-α, IFN-γ and IL-6 in triggering ET release by G-MDSCs, other molecules that are enriched in lupus serum, such as immune complexes cannot be exempted. In support of this statement, Carmona-Rivera *et al.* have shown that immune complexes containing matrix

metalloproteinase-9 (MMP)-9 and anti-MMP2 enhance NETosis in LDGs (Carmona-Rivera, Zhao et al. 2015).

Although the molecular events leading to NET release are not fully elucidated, data suggest that are critically dependent upon the type of the "inflammatory" stimulus (Goldmann and Medina 2012). To this end, PMA-mediated NET release was shown to require simultaneous activation of autophagy pathway as well as NADPH oxidase and ROS production (Remijsen, Vanden Berghe et al. 2011). Our findings did not indicate any differences in autophagy activation between NZB/W F1 and B6 serum-treated $Ly6G^+$ cells. Although we cannot exclude a possible role of autophagy in ET formation by G-MDSCs, our results clearly demonstrate that generation of ROS is indeed necessary for ET release, since ROS scavenging reduces ET generation by $Ly6G^+$ cells.

The role of ROS in SLE pathogenesis is not clear to date. Conflicting data present an increased production of ROS by PMNs from SLE patients whereas others demonstrate an association between decreased ROS generation by SLE-PMNs and disease severity (Perazzio, Salomao et al. 2012; Bengtsson, Pettersson et al. 2014). These differences could reflect the heterogeneity of the PMN populations as well as different therapeutic regimens that PMNs have been exposed to (Fuchs, Abed et al. 2007; Fridlender, Sun et al. 2009; Zhang, Majlessi et al. 2009; Perazzio, Salomao et al. 2012; Pillay, Tak et al. 2013; Bengtsson, Pettersson et al. 2014). Besides, these conflicting data could also reflect the different sources that ROS are released from (Holmstrom and Finkel 2014). Our results provide evidence for increased production of ROS by granulocytic cells exposed to lupus serum as well as to inflammatory cytokines that have been closely linked to lupus pathogenesis. Actually, scavenging of

total ROS inhibited ET release by G-MDSCs. However, sole inhibition of NADPH oxidase activity by DPI (20 μM) slightly attenuated ROS production in NZB/W F1 serum-treated B6 naive-derived BM Ly6G$^+$ cells, suggesting that ROS production in Ly6G$^+$ cells might not require NADPH activity. This is supported by the observations of a study in lupus-prone MRL/lpr mice that are deficient in Cybb (cytochrome b-245, beta polypeptide, also known as Nox2) and which therefore lack functional NADPH oxidase. Of interest these mice develop a worsening lupus phenotype when compared to mice that are not deficient in Cybb (Campbell, Kashgarian et al. 2012). Thus, we looked at mitochondrial ROS production, that has been already associated with chronic inflammatory conditions (Yu and Bennett 2014). Indeed, our results demonstrate an important role of mitochondria in ROS production by Ly6G$^+$ cells under the lupus microenvironment. In support, two recent studies showed that basophils produce mitochondrial ROS that is required for generation of ETs in response to IL-3 an allergic enriched cytokine (Morshed, Hlushchuk et al. 2014) and that mitochondrial ROS are capable to drive NETosis after ribonucleoprotein-containing immune complexes (RNP ICs) stimulation *in vitro* and in the pro-inflammatory LDG subset in lupus and CGD subjects *ex vivo (Lood, Blanco et al. 2016)*.

One common characteristic of MDSCs in cancer, infections, and several autoimmune diseases is their suppressive activity. As such, delineating MDSCs functional role in SLE where the inflammation is unresolved and MDSCs were found eliminated appeared very interesting, if not obligatory. Of interest our study revealed that G-MDSCs in lupus not only are they dysfunctional, but actually are characterized by new properties; they are immunogenic and promote T cell activation and proliferation. M-MDSCs on the other hand appeared suppressive. The ability of M-MDSCs to suppress T cells in contrast to G-MDSCs was also described in NZB/W F1 mice by

Der *et al*. (Der, Dimo et al. 2014). Similarly, a study in MRL-Faslpr lupus prone mice has shown that $CD11b^{+}Gr\text{-}1^{low}$ MDSCs suppressed T cell proliferation via Arg1 activity (Iwata, Furuichi et al. 2010). However none of these studies has attributed this altered MDSC phenotype to the inflammatory environment of lupus. Herein, we show that this newly acquired property of MDSCs is driven by the inflammatory milieu of lupus. Notably, the suppressive assays that contained total MDSCs showed that their effect on T cells was immunogenic. We postulate that the lupus microenvironment drives ET release by G-MDSCs that in turn stimulate M-MDSCs to differentiate and become pro-inflammatory contributing to perpetuation of lupus. Whether this is the case or if the immunogenic effect of G-MDSCs simply masks the suppressive effect by M-MDSCs needs to be further investigated.

Overall, this study establishes a critical role of G-MDSCs in the pathogenesis of lupus and provides new insights into the deregulation of the tolerogenic mechanisms that is critical for the immunogenicity and perpetuation of autoreactive responses. Understanding the underlying causative mechanisms that drive the elimination of G-MDSC regulatory compartment will provide opportunities for the development of targeted and desirably more efficient therapeutic methods for patients with systemic autoimmune responses.

7. FUTURE DIRECTIONS

Our data reveal a newly identified defect in the G-MDSC regulatory compartment in lupus. Moreover, we provide evidence for the key role that the inflammatory environment of lupus plays in the regulation of MDSCs function and fate. Based on our findings many relevant questions have been raised that could be further explored. Such questions of scientific interest are discussed below.

- **What is the effect of G-MDSCs ETosis on adaptive immune responses in lupus?**

Previous studies have shown that neutrophils can trigger adaptive immune responses through NETosis, by which the released self DNA in complex with antimicrobial peptides can be immunogenic. In our study we show that G-MDSCs promote T cell proliferation and activation in lupus. Whether this property of G-MDSCs is mediated by ETosis remains to be answered. In the future, we seek to examine how MDSCs control T cell differentiation and whether they affect B cell responses on a T cell-independent manner as well. This can be addressed as follows;

i) First of all, to examine this we will see if G-MDSC mediated T cell activation and proliferation is due to ETosis. For this purpose we are going to perform the suppression assay we have already used, after pre-treating G-MDSCs with NAC, in order to eliminate ETosis. More specifically we will isolate $CD11c^{-}$ $CD11b^{high}Ly6G^{+}$ G-MDSCs from the BM and/or spleen of NZB/W F1 mice and $CD4^{+}CD25^{-}$ T cells from LNs of the same mice. G-MDSCs will be treated with NAC and T cells will be labeled with CFSE prior to co-culture at 1:1 ratio, for 5 d in the presence of anti-CD3/CD28 polyclonal stimulation. $CD4^{+}$ T cell proliferation and activation will be then monitored by FACS analysis of CFSE dilution and CD25/CD44 expression respectively.

ii) To examine how G-MDSCs control T cell responses we are going to purify by sorting naive $CD4^+CD25^-CD62L^+$ T cells from the spleen of NZB/W F1 pre-diseased and diseased mice. We will first label naive T cells with CFSE and then culture them under three different conditions; a) Th1 polarization medium containing IL-12, IFN-α and anti-IL-10, b) Th2 polarization medium containing IL-4 and anti-IFN-γ and c) Th17 polarization medium containing IL-6, TGFβ, anti-IFN-γ and IL-4, in the presence of anti-CD3/CD28. T cells alone with one of the three culture media will be the control. For each one of the conditions we will add $CD11c^-CD11b^{high}Ly6G^+$ G-MDSCs isolated from the BM and/or spleen of the same mouse to assess whether G-MDSCs promote Th1, Th2 or Th17 responses. The response of T cells will be monitored by measuring certain cytokines in the culture supernatant 5 d after co-culture by ELISA. The cytokines that we will measure to account for Th1, Th2 or Th17 response will be the following a) IFN-γ b) IL-4,IL-5,IL-10 and IL-13 and c) IL-17, respectively. Alternatively, instead of performing ELISA in the supernatants of the culture we could perform an ELISpot assay, as a more sensitive method to detect cytokine production at a single cell level.

According to the result, we will continue to assess which molecule mediates this effect, by selectively blocking one cytokine from the culture medium each time. In this way, we will be able to identify cytokines secreted by G-MDSCs that possibly drive naive T cells into Th1, Th2 or Th17 differentiation.

iii) In order to assess how lupus G-MDSCs affect B cell differentiation we will set an assay where we will co-culture the two subsets and then monitor IgM and IgG secreting B cells, by ELISpot assay. $CD11c^-CD11b^{high}Ly6G^+$ G-MDSCs will be flow-sorted from the BM and/or the spleen and $B220^{high}CD19^{high}$ B cells from the spleen of NZB/W F1 pre-diseased. The two cell populations will be co-cultured at different ratios in the presence or absence of rIFN-α and anti-CD40, that will be

used as positive control. We will then harvest cells at two difeerent time points, 3 and 5 d, and assess IgM and IgG secreting B cells by ELISpot. This setting would give insight on the direct act of G-MDSCs on B cells. Otherwise, in order to address whether G-MDSCs have an effect on B cells on a T-cell dependent way, we would either culture all the three populations in the same system or we would use the supernatant of G-MDSCs/T cell cultures to trigger B cell isolated from NZB/W F1 mice and then assess IgG and IgM secretion. The latter case pre-requisites of course to have soluble factors triggering B cells in the absence of G-MDSCs and/or T cells.

- **How does the lupus inflammatory environment affect MDSC phenotype? Identify lupus MDSC (a) gene profile and (b) differentiation.**

MDSCs receive signals from the inflammatory environment to express certain molecules that are involved in specific signaling pathways in order to be activated and maintain their suppressive function (Condamine and Gabrilovich 2011). Since our results show that lupus G-MDSCs are not suppressive, but on the other hand they promote T cell responses, we hypothesize that this newly acquired properties are correlated with an altered transcriptional activity. To identify molecules that are altered in lupus G-MDSCs, we plan to follow the steps described below.

i) At one hand, we will isolate G-MDSCs from a) NZB/W F1 female pre-diseased and diseased mice, b) NZB/W F1 male naive and CFA-immunized mice, c) EAE mice and d) NZB/W F1 CFA-immunized mice (as long as we show that these are suppressive). We will then use Affymetrix genechip microarrays to check gene expression levels. Analysis of results will give the opportunity to define transcripts that are deregulated in NZB/W F1 mice; in turn, we will identify the canonical pathways that will be associated with the

defined pathways by performing Ingenuity Pathway Analysis (IPA). We plan to run the same experiment for M-MDSCs which despite being suppressive *in vitro*, they are apparently not sufficient to control the inflammatory responses *in vivo.*

ii) In addition to gene activity, lupus MDSCs might also differ from common MDSCs in the expression of surface markers that are relevant to their maturation and activation status, due to deregulation at post-transcriptional levels. Therefore, we could assess such markers expression on the surface of G-MDSCs and M-MDSCs isolated from the aforementioned mouse groups. Among the markers expressed on MDSCs and their descendants (mature granulocytes and monocytes) we selected the most relevant to their function and these are given in Table 2.

Table 2: Surface markers for characterization of MDSCs differentiation status.

A/A	Protein	Description/ Role
1	**MHC Class II**	Major histocompatibility complex) class II, Antigen-presentation to T cells.
2	**CD11c**	Integrin alpha X (complement component 3 receptor 4 subunit) chain protein. Induces cellular activation, triggers neutrophil respiratory burst.
3	**CD80**	Cluster of Differentiation 80 (B7-1). Co-stimulatory molecule necessary for T cell activation and survival.
4	**CD86**	Cluster of Differentiation 86 (B7-2). Co-stimulatory molecule necessary for T cell activation and survival.
5	**CD40**	Cluster of Differentiation 40. Co-stimulatory molecule on the surface of APCs, required for their activation.
6	**CD15**	Cluster of Differentiation 15. Mediates phagocytosis and chemotaxis.
7	**F4/80**	Member of the adhesion GPCR family. Mainly expressed on mature macrophages.
8	**CCR5**	C-C chemokine receptor type 5 (also known as CD195).
9	**CXCR4**	C-X-C chemokine receptor type 4 (also known as CD184). Specific receptor for stromal-derived-factor-1 (SDF-1 also called CXCL12).
10	**CD33**	Also known as Siglec-3. Myeloid-specific transmembrane receptor.
11	**CD124**	Interleukin-4 receptor.

12	**CD66b**	Cluster of Differentiation 66b. Mainly expressed on activated granulocytes. Involved in cell adhesion, cell migration and pathogen binding.
13	**CD14**	Cluster of Differentiation 14. Co-receptor responsible for the detection of LPS.
14	**M-CSFR (CD115)**	Macrophage colony-stimulating factor receptor (also known as CD115). Receptor for colony stimulating factor 1 (CSF1).
15	**G-CSFR (CD114)**	Granulocyte colony-stimulating factor receptor. Expressed on precursor cells in the bone marrow. Initiates cell proliferation and differentiation into mature neutrophilic granulocytes and macrophages.
16	**GM-CSFR (CD116)**	Granulocyte macrophage colony-stimulating factor receptor. Normally located on myeloblasts and mature neutrophils. Stimulates the production of white blood cells.
17	**CD244**	Cluster of Differentiation 244. Mainly expressed on NK cells. Mediates non-MHC restricted killing. Highly expressed on G-MDSCs too.

- **How does the lupus inflammatory environment affect G-MDSC ETosis?**

Our data revealed that the inflammatory environment of lupus promotes ETosis in G-MDSCs. Moreover, among the various inflammatory molecules that the lupus serum contains we identified that IFN-α, IFN-γ and IL-6 can promote ETosis. Of note, the serum from NZB/W F1 young pre-diseased (without proteinuria) mice could also drive ET formation, however to a lesser extent. Therefore, we assume that there are several soluble factors in the serum of the NZB/W F1 mice that contribute to the elimination of G-MDSCS through ETosis before tissue damage. In order to identify such molecules that contribute to this phenomenon, we plan to do the following;

i) Initially we will quantify spontaneous ET release in mice of younger age than NZB/W F1 young mice (3-4 mo old) contained in our analyses already. Results will be compared with already existing groups; NZB/W F1 pre-diseased mice (3-4 mo old) and NZB-W F1 diseased mice (>7mo old).

ii) Next, we will collect blood serum from NZB/W F1 of different age starting from 8 wk old mice, every three weeks and not more often, in order to have all constituents of the blood returned to normal. Blood collection will last at least

until proteinuria appears, although this would be time-consuming, since this model develops spontaneously proteinuria not earlier than the 6th month of age usually. In parallel, we will be collecting urea samples to be analyzed for protein levels. After samples collection, we will perform bead array immunoassay to examine the cytokine/chemokine profile in the serum of mice at different ages. Comparison of the cytokine/chemokine profile of pre-diseased mice with diseased mice will allow the identification of inflammatory molecules that are first increased in NZB/W F1 mice, early on before the first traces of proteins appear in the urea samples. These molecules will be set as candidates for driving ETosis in the context of lupus.

iii) To identify the candidate molecules effect on ET release by G-MDSCs, we plan to treat G-MDSCs isolated from B6 naive mice and/or NZB/W F1 male mice with each one of these separately or in combinations and assess ETosis by immunofluoresence. Repeating the same assay with a gradient of concentrations will allow to identify whether the effect of a molecule on ETosis is dose-dependent.

iv) Moreover, for molecules that will be identified as drivers of ETosis, we plan to use specific inhibitory antibodies in a culture system of G-MDSCs isolated from B6 naive mice and/or NZB/W F1 male mice, treated with NZB/W F1 diseased-derived serum. Quantification of ET release will help identifying molecules which mediate ET formation by G-MDSCs.

v) According to our data that IFN-α, IFN-γ as well as IL-6 can promote ETosis, we believe that we will identify more than one molecules that early on can promote ETosis in lupus mice. Thus, after performing the above experiments we plan to select the most potent mediators of ETosis and perform *in vivo* administration of neutralizing antibodies against the respective cytokines.

Administration will start very early at the time points that the bead array immunoassay will nominate and different combinations will be tried. One group of the mice involved in the experiment will be followed up for proteinuria and survival and others will be sacrificed at different time points to examine spontaneous ET release. Moreover the serum of the treated mice will be collected at different time points to assess their potential to drive ETosis in G-MDSCs isolated from B6 naive mice and/or NZB/W F1 male mice with immunofluoresence.

- **What is the source of ROS mediating ETosis in lupus G-MDSCs?**

It is known that ROS production is essential for NET formation (Remijsen, Vanden Berghe et al. 2011). However ROS can be produced by various systems in the cell (Holmstrom and Finkel 2014) and studies have shown that different sources of ROS can be responsible for extracellular trap formation (Morshed, Hlushchuk et al. 2014). In our analysis, we showed that the inflammatory environment of lupus leads to increased production of ROS by $Ly6G^+$ cells, and more specifically mitochondrial ROS. Moreover, we showed that NAC, a ROS scavenger, significantly impeded ETosis in $Ly6G^+$ cell treated with NZB/W F1 diseased serum. Identifying the source of ROS mediating ETosis, would help interfere this pathway and maybe prevent ET formation. Therefore, we plan to perform some more experiments in order to examine the contribution of NADPH and mitochondria activity on ETosis by G-MDSCs.

i) At first, we plan to see if mitochondria is the major source of ROS produced after stimulating $Ly6G^+$ cells with the inflammatory environment of lupus. For this purpose, we will isolate $Ly6G^+$ cells from the BM of B6 naive mice, pre-treat them with the flavoprotein inhibitor MitoQ, to block ROS generation by mitochondria and then stimulate them with serum isolated from NZB/W F1

diseased mice. Ly6G$^+$ cells treated with FBS or B6 naive serum will be used as controls. Then, with the use of DCF, a cellular reactive oxygen species detection assay kit, we will measure total ROS production by FACS analysis. Cells stimulated with the sera mentioned above, but without being pre-treated with MitoQ will be used as controls. Comparison of the two sets of experimental groups (MitoQ-treated and untreated) will show whether the increased ROS production that we observed in NZB/W F1 diseased serum-treated Ly6G$^+$ cells is mitochondria-derived, as long as treatment of these cells with MitoQ diminishes the detection of total ROS levels.

ii) Continuing working on the hypothesis that mt-derived ROS mediates ETosis, we are planning to assess ETosis with the commonly-used protocol used in this study, but this time after pre-treating cells with MitoQ. Quantitation of ET formation by Ly6G$^+$ cells treated with NZB/W F1 diseased serum in combination or not with MitoQ will reveal whether blocking mitochondria ROS production eliminates ETosis.

iii) To further confirm how the inflammatory environment of lupus affects NADPH and mitochondria activity simultaneously with ET formation, we are going to validate the enzymatic activity of NADPH by NBT assay and superoxide levels in the mitochondria by fluoroprobe MitoSOX Red. Cells will be stained with the DNA staining dye DAPI to correlate ROS production with ETosis. Combination of confocal laser scanning and differential interfere contrast (DIC) microscopy will be used to visualize the enzymatic activity of NADPH and mitochondria in ET forming cells. This assay will give us the opportunity to see how the two different sources of ROS mediate ETosis in Ly6G$^+$ cell treated with NZB/W F1 diseased serum.

iv) In order to further explore the role of mitochondrial ROS production on ETosis driven by the inflammatory environment of lupus, we plan to use Ncf1 knockout mice. These mice carry a mutation at *Ncf1* gene encodes for the neutrophil cytosolic factor 1 (NCF1/p47-pho), a component of the NADPH oxidase complex that is involved in the production of reactive oxygen species (ROS), (Hultqvist, Olofsson et al. 2004). Therefore, Ncf1 knockout mice have undetectable levels of NADPH-derived ROS. We will isolate $Ly6G^+$ cells from the BM of Ncf1 knockout mice and treat them with NZB/W F1 diseased-derived serum in the presence or absence of MitoQ reagent to observe and quantify ETosis by immunofluoresence. Serum from B6 naive mice will be used as control. The same set of stimulation will be used for treating $Ly6G^+$ cells isolated from B6 wild type mice, which is an appropriate control for Ncf1 knockouts as the latter have been reproduced on a B6 genetic background. We will the observe and measure ETosis by confocal laser scanning microscopy in order to evaluate; a) the potential of the inflammatory environment of lupus to induce ETosis, in $Ly6G^+$ cells that lack NADPH activity and b) the role of mt-derived ROS in ETosis by comparing ETosis in Ncf1 knockout-derived $Ly6G^+$ cells treated with NZB/W F1 serum plus MitoQ with the respective cells that were not treated with MitoQ, but only with serum.

v) Finally, in case our hypothesis that mt ROS mediates ET formation G-MDSCs of lupus mice is likely, we would try to administer MitoQ *in vivo* in NZB/W F1 mice and monitor proteinuria by measuring protein levels in their urea and inflammatory mediators levels by ELISA. MitoQ administration would start in one group of mice early from 8 wk old mice to evaluate its potential to prevent disease development. In a second group of mice we will start MitoQ administration later, starting from the 5^{th} month of age the earliest when proteinuria will have been already established (>=100 ng/dl), in order to

estimate the MitoQ ability to eliminate inflammation and ameliorate proteinuria. MitoQ will be administered twice a week, i.v. at a dose of 100 nM/mouse for both groups for at least 30 d. Urea protein levels and serum anti-nuclear and anti-DNA levels will be measured at the beginning of the treatment and will be repeated three times a week for proteinuria and once a week for autoantibody responses.

- **What is the interplay between M-MDSCs and G-MDSCs?**

According to our study, M-MDSCs isolated from NZB/W F1 mice are suppressive *in vitro* in contrast to G-MDSCs that promote T cell proliferation and activation. However, when these two subsets are co-cultured together with T cells, the final outcome is MDSC-induced T cell proliferation and activation. Whether this is due to that the immunogenic properties of G-MDSCs mask the suppressive effect derived from M-MDSCs or that G-MDSCs have an effect on M-MDSCs and promote their differentiation into a more mature, immunogenic state remains unknown. We aim to examine what is the interplay between the two subsets, using the following strategy;

i) The suppression assay we have been using in this study allowed contact of all cell subsets in the same culture dish well. At first site we are willing to repeat the suppression assay using a transwell culture system, to verify whether G-MDSCs would have the same effect on M-MDSCs suppressive activity if separated by a semipermeable membrane. More analytically, we will sort $CD11c^{-}CD11b^{high}Ly6\text{-}G^{+}$ G-MDSCs and $CD11c^{-}CD11b^{high}Ly6C^{+}$ M-MDSCs from the spleen of NZB/W F1 mice and $CD4^{+}CD25^{-}$ T cells from their LNs. Simultaneously with sorting, M-MDSCs will be analyzed for expression of MHC Class II, CD80, CD86, CD40 and CD11c expression on their surface before put in culture. We will label T cells with CFSE and will place them at the apical

side of the transwell together with M-MDSCs. in the presence of anti-CD3/CD28 stimulation. G-MDSCs will be placed in the basolateral side and cells will be left in the culture for 5 d. Then we will harvest cells from the culture and assess a) T cell proliferation by CFSE dilution and activation by CD25/CD44 expression and b) MHC Class II, CD80, CD86, CD40 and CD11c expression on the surface of M-MDSCs. Along with cell analyses we will measure NO levels in the culture medium with a Griess Reagent Kit for Nitrite Determination, assuming that M-MDSCs use NO to suppress T cells. M-MDSCs cultured with anti-CD13/CD28 stimulated T cells only will be used as a control.

ii) If our hypothesis that G-MDSCs alter M-MDSCs suppressive activity is confirmed, then we would try to eliminate G-MDSCs *in vivo* in NZB/W F1 lupus mice. This would possibly have dual impact; elimination of the inflammatory environment-induced ETosis in G-MDSCs on the one hand and re-establishment of immune tolerance by M-MDSCs on the other hand. For this purpose, we will administer anti-Ly6G *in vivo* in NZB/W F1 mice and assess disease activity by measuring urine protein levels and serum anti-nuclear and anti-DNA antibody levels. Given the fact that NZB/W F1 lupus-like model is spontaneous there are some limitations for this *in vivo* experiment, concerning the initiation and duration of the treatment, as well as the correct control group. However, we thought of starting with two groups. One group with NZB/W F1 young mice (3-5 mo old) at the beginning of proteinuria and one with older mice (6-9 mo old) with established proteinuria for at least one month. We will follow a twice-a-week treatment in both groups by injecting a rat anti-mouse Ly6G mAb i.p. at a moderate dose of 1 mg/mouse or isotype control. Urine protein levels will be quantified by a visual dipstick test three times per week. Mice will be bled once a week and serum anti-nuclear and

anti-DNA levels will be quantified by ELISA. This setting will allow to explore the potential of tolerance re-establishment in the absence of the defective and immunogenic G-MDSC regulatory compartment in lupus.

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APPENDIX I

Elimination of granulocytic myeloid-derived suppressor cells in lupus-prone mice due to ROS-dependent extracellular trap formation.

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Abstract

Objective. Emerging evidence supports a crucial role of myeloid-derived suppressor cells (MDSCs) in the regulation of autoimmune diseases, however their role in systemic lupus erythematosus (SLE) remains unknown. In this study we address the role of MDSCs in the pathogenesis of SLE.

Methods. The NZB/W F1 lupus-prone mouse model was used to assess MDSC phenotype by flow cytometry and function through *in vitro* T-cell proliferation assay and real-time quantitative PCR. Extracellular Trap formation was evaluated by immunofluoresence and confocal microscopy. The production of reactive oxygen species (ROS) by $Ly6G^+$ cells was determined by FACS analysis.

Results. Herein, we demonstrate an impaired expansion and defective function of MDSCs in the lymphoid organ of NZB/W F1 lupus-prone mice with established disease that involved predominantly the granulocytic MDSC cell subset (G-MDSCs). More specifically, we found increased elimination of G-MDSCs due to extracellular trap (ET) formation driven by the inflammatory milieu of lupus and we demonstrate a role of cytokines such as IFN-α, IFN-γ and IL-6 in this process. Induction of ET release by G-MDSCs was mediated by production of ROS, since inhibition of ROS generation significantly reduced ET release.

Conclusion. Collectively, our findings reveal the elimination of a crucial regulatory immune cell subset in SLE microenvironment and provide new insights into the pathogenetic mechanisms of the disease.

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APPENDIX II

In vitro suppression of CD4$^+$ T cell responses by murine and human myeloid-derived suppressor cells.

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Abstract

Myeloid-derived suppressor cells (MDSCs) are myeloid precursors of macrophages, dendritic cells and neutrophils with a prominent role in the regulation of immune responses in cancer, infection and autoimmunity. Herein, we describe a protocol for the isolation of murine and human MDSCs and the assessment of their ability to suppress CD4$^+$ T cell responses *in vitro*.

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