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Arginase 1$^+$ IL-10$^+$ polymorphonuclear myeloid-derived suppressor cells are elevated in patients with active pemphigus and correlate with an increased Th2/Th1 response

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Abstract

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells, which are characterized by their capability to suppress T-cell responses. While MDSCs have been traditionally associated with cancer diseases, their role as regulators of autoimmune diseases is emerging. Pemphigus is a chronic autoimmune blistering skin disease characterized by dysregulated T-cell responses and autoantibody production. The role of MDSCs in pemphigus disease has not been defined yet. The aim of this study was to characterize MDSCs in pemphigus patients and to dissect their relationship with CD4$^+$ T-cell subsets and clinical disease assessments. For this purpose, we performed a cross-sectional analysis of 20 patients with pemphigus. Our results indicate that a population of CD66b$^+$CD11b$^+$ polymorphonuclear-like MDSCs (PMN-MDSCs) is expanded in the peripheral blood mononuclear cell fraction of pemphigus patients compared to age-matched healthy donors. These PMN-MDSCs have the capability of suppressing allogeneic T-cell proliferation *in vitro* and show increased expression of characteristic effector molecules such as arginase I and interleukin-10. We further demonstrate that PMN-MDSCs are especially expanded in patients with active pemphigus, but not in patients in remission. Moreover, MDSC frequencies correlate with an increased Th2/Th1 cell ratio. In conclusion, the identification of a functional PMN-MDSC population suggests a possible role of these cells as regulators of Th cell responses in pemphigus.

KEYWORDS
autoimmune skin blistering disease, autoimmunity, desmoglein, MDSC, T helper cells

Abbreviations: ACTB, Beta-actin; ARG1, Arginase I; Dsc, Desmocollin; Dsg, Desmoglein; EAE, Experimental autoimmune encephalomyelitis; GVHD, Graft-versus-host disease; IFNγ, Interferon gamma; IL-10, Interleukin-10; IL-13, Interleukin-13; IL-2, Interleukin-2; IL-4, Interleukin-4; IL-4Rα, Interleukin-4 receptor alpha; iNOS, Inducible nitric oxide synthase; MDSCs, Myeloid-derived suppressor cells; MHC, Major histocompatibility complex; M-MDSCs, Monocytic myeloid-derived suppressor cells; NO, Nitric oxide; PBMCs, Peripheral blood mononuclear cells; PDAI, Pemphigus disease area index; PF, Pemphigus foliaceus; PMN-MDSCs, Polymorphonuclear-like myeloid-derived suppressor cells; PMNs, Polymorphonuclear neutrophils; POLR2A, Polymerase (RNA) II (DNA directed) polypeptide A; PV, Pemphigus vulgaris; RA, Rheumatoid arthritis; ROS, Reactive oxygen species; SLE, Systemic lupus erythematosus; Tfh, T follicular helper; Tfreg, T follicular regulatory; TGF-β, Transforming growth factor beta; Th, T helper; Treg, T regulatory.

1 | INTRODUCTION

Myeloid-derived suppressor cells (MDSCs) are a population of myeloid cells first described in cancer patients, where they show the capability of potently suppressing T-cell responses.[1] Beyond oncological settings, myeloid cells can influence T- and B-cell development and function with implications for other disease conditions such as sepsis and autoimmunity.[2,3]

Human MDSCs are divided into two main groups, granulocytic or polymorphonuclear-like MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs).[4] These cells are phenotypically and morphologically similar to neutrophils and monocytes, respectively. In humans, PMN-MDSCs share common surface markers with conventional polymorphonuclear neutrophils (PMNs), namely $CD11b^+$, $CD15^+$, $CD33^+$ and $CD66b^+$, but can be separated from PMNs through density separation: while PMN-MDSCs are found in the low-density Ficoll-gradient fraction of peripheral blood mononuclear cells (PBMCs), PMNs appear in the high-density fraction. Conventional monocytes are defined by $CD11b^+$, $CD14^+$, $CD15^-$, $CD33^+$ and HLA-DR^+, whereas M-MDSCs are HLA-$DR^{-/low}$.[5] According to their different morphologies, PMN-MDSCs and M-MDSCs employ different mechanisms of immunosuppression. While the suppressive function of PMN-MDSCs is mediated by production of reactive oxygen species (ROS) and arginase I (ARG1), M-MDSCs act through the production of nitric oxide (NO).[6,7]

Through these features, MDSCs are able to target different components of the adaptive immune response. MDSCs can potently suppress T-cell responses by depriving lymphocytes of arginine through ARG1 and by generating oxidative stress through ROS and NO.[8,9] Moreover, MDSCs are able to modulate T-cell response by altering the balance between proinflammatory and regulatory T helper (Th) cell subsets through production of cytokines such as interleukin-10 (IL-10) and transforming growth factor beta (TGF-β).[10,11] Both PMN-MDSCs and M-MDSCs can also affect B-cell responses by suppressing B-cell proliferation and antibody production.[12,13] These immunoregulatory properties support the key role of MDSCs as regulators of the immune system in different human pathologies. While in cancer and infectious diseases, the presence of MDSCs has been associated with poor clinical outcome, the role of MDSCs in autoimmune diseases is more controversial. Several studies imply that the accumulation of these suppressive cells might be beneficial, but other reports associated MDSCs with disease progression in autoimmunity. For instance, in a mouse model of systemic lupus erythematosus (SLE), MDSCs ameliorated autoimmunity by modulating the B-cell response.[14] However, studies in SLE patients associated MDSCs with increased Th17 responses and disease severity.[15] Similarly, MDSCs in the synovial fluids of patients with rheumatoid arthritis (RA) could effectively suppress T-cell activity;[16] however, their increased frequency correlated with disease progression.[17] Overall, most studies point to a regulatory role of MDSCs in autoimmune settings, but their specific mechanisms of action seem to differ in each disease and require further clarification.

Pemphigus is a rare chronic autoimmune blistering disease characterized by the formation of intraepidermal splits and loss of keratinocyte adhesion (also known as acantholysis), which usually manifests between the age of 40 and 65.[18] Acantholysis is caused by autoantibodies, the majority of which target structural proteins of epidermal desmosomes known as desmoglein (Dsg) 1 and Dsg3.[19] There are two main clinical variants of pemphigus, defined by the predominant anti-Dsg autoantibody profile. Pemphigus vulgaris (PV) shows primarily mucosal lesions like enanthema and erosions, mainly localized in the buccal mucosa with possible involvement of nasal, pharynx and genital mucosa and is characterized by an anti-Dsg 3 autoantibody profile. In pemphigus foliaceus (PF), lesions are restricted mainly to the seborrhoeic areas of the skin as flaccid blisters and erosions, and the predominant autoantibody present is directed against Dsg 1.[20] Pemphigus diseases and its variants like paraneoplastic pemphigus or pemphigus vegetans can also be caused by autoantibodies directed against other desmosomal components than Dsgs, such as desmocollin (Dsc)1, Dsc2 and Dsc3.[21–24]

According to recent studies, the production of Dsg-specific autoantibodies by B cells is driven by Dsg-specific autoreactive Th and T follicular helper (Tfh) cells.[25,26] Interleukin-4 (IL-4) producing Dsg autoreactive Th2 cells have been described in acute and chronic patients, while Interferon gamma (IFNγ) producing Th1 cells seem to be predominant in remitting patients.[27,28] A dysfunction in IL-10 secreting Treg cells might also be implied in the loss of tolerance against Dsgs.[29] More recent studies also suggest an Important role of Th17 and Tfh cell populations in pemphigus pathogenesis.[26,30–32]

Given the established role of MDSCs as regulators of T-cell responses, we hypothesized that chronic inflammation in pemphigus patients modulates MDSCs and downstream T-cell responses.

2 | MATERIALS AND METHODS

2.1 | Study population

The study was conducted at the University Hospital of Dermatology in Tübingen, Germany. Informed written consent was obtained from all subjects included in the study, and all methods were approved by the local ethics committee. Aim of the study was to explore whether MDSCs were expanded in pemphigus patients compared with healthy donors. The study population consisted of 20 pemphigus patients (13 females and 7 males) with ages ranging from 36 to 85 years. 18 patients were diagnosed with pemphigus vulgaris and two patients with pemphigus foliaceus (Table 1).

The clinical diagnosis of pemphigus was confirmed by patients' records of histopathology (suprabasal acantholytic blisters), direct immunofluorescence microscopy (epidermal intercellular IgG and/or C3 deposits in perilesional skin), and detection of autoantibodies against Dsg1 and/or Dsg3. The presence of IgG autoantibodies against Dsg1 or Dsg3 in patient's sera at the time of study sampling was evaluated by anti-Dsg1- and anti-Dsg3-ELISA according

TABLE 1 Clinical characteristics of study subjects

Subject	Gender	Age	Diagnosis	Current therapy	Anti-Dsg1 IgG (RU/ml)	Anti-Dsg3 IgG (RU/ml)	Clinical status	PDAI score
P#1	M	60	PV	AZA, DAP, PD	0	0	Chronic active	7
P#2	M	79	PV	AZA, PD	0	106	Chronic active	2
P#3	M	50	PV	PD	87	89	Chronic active	14
P#4	F	70	PV	PD	0	0	Chronic active	2
P#5	F	55	PV	MTX, PD	20	200	Chronic active	2
P#6	F	69	PV	AZA, PD	11	0	Remission	0
P#7	F	48	PF	AZA	17	3	Chronic active	3
P#8	M	56	PV	CE, DAP	0	7	Remission	0
P#9	F	68	PV	AZA, PD	51	40	Chronic active	2
P#10	M	74	PV	CE, PD	2	66	Chronic active	2
P#11	F	64	PV	PD	114	171	Chronic active	7
P#12	F	40	PV	AZA	NA	NA	Chronic active	1
P#13	M	40	PV	CE, RTX	0	15	Chronic active	1
P#14	F	51	PV	AZA, PD	0	200	Remission	0
P#15	M	82	PV	AZA	10	9.2	Chronic active	3
P#16	F	36	PV	AZA, PD	14	14	Chronic active	5
P#17	F	85	PF	PD	172	0	Chronic active	9
P#18	M	67	PV	AZA	0	19	Remission	0
P#19	F	46	PV	CE	3	176	Remission	0
P#20	F	57	PV	DAP	0	11	Remission	0

Note: Clinical status as classified in the Materials and methods section at the time of sample procurement.

Abbreviations: AZA, Azathioprin; CE, CellCept (mycophenolate mofetil); DAP, Dapson; Dsg, desmoglein; MTX, methotrexate; NA, not available; PD, prednisone; PDAI, pemphigus disease area index; PF, pemphigus foliaceus; PV, pemphigus vulgaris; RTX, Rituximab.

to the manufacturer's protocol (Euroimmun). Based on a clinical specificity of ≥99%, the cut-off of both immunoassays was set at 20 RU/ml.[33]

At the time of blood sampling, all pemphigus patients received regular treatment with systemic immunosuppressants. Clinical activity was assessed using the Pemphigus Disease Area Index (PDAI) score.[34] In our cohort, all patients with active disease scored a PDAI <15. Patients with remittent pemphigus had not experienced new mucosal blisters/erosions for 6 months or more before the study and were given a PDAI score of 0.

Control subjects (8 females and 11 males) were from 35 to 70 years of age. Exclusion criteria included acute and chronic infections, malignancies, autoimmune diseases, obesity, pregnancy as well as prior history of autoimmune or non-autoimmune inflammatory disorders of the skin).

2.2 | Flow cytometry and cell isolation

Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized blood samples by Ficoll density gradient sedimentation with Biocoll separating solution (Merck KGaA) and washed twice in RPMI 1640 medium (Merck KGaA). Trypan blue staining solution (Sigma-Aldrich) at 0.5% differentiated between viable and non-viable cells and showed a viability of >90% for all cells used in this study. After Ficoll density gradient sedimentation, PMN-MDSCs were characterized as $CD11b^+$, $CD14^-$, $CD33^+$ and $CD66b^+$, M-MDSCs as $CD11b^+$, $CD14^+$, $CD33^+$ and $HLA\text{-}DR^{-/low}$[5] (as depicted in Figure 1A). Th cells were characterized as $CD3^+$, $CD4^+$, $CD45RA^-$ and $CXCR5^-$, while Tfh cells were defined as $CD3^+$, $CD4^+$, $CD45RA^-$ and $CXCR5^+$. The different Th and Tfh subsets were characterized as following: Th1 or Tfh1 cells: $CXCR3^+$, $CCR6^-$; Th2 or Tfh2 cells: $CXCR3^-$, $CCR6^-$; Th17 or Tfh17 cells: $CXCR3^-$, $CCR6^+$; T regulatory (Treg) or T follicular regulatory (Tfreg) cells: $CD25^{+/high}$, $CD117^{-/low}$[26,35,36] (as depicted in Figure 3A).

For PMN-MDSC isolation, PBMCs were obtained from the low-density fraction of Ficoll density gradient sedimentation and labelled with anti-CD66b-FITC followed by two sequential anti-FITC magnetic bead separation steps (Miltenyi Biotec), according to the manufacturer's protocol. Conventional PMNs were isolated from the high-density fraction of Ficoll density gradient sedimentation followed by erythrocyte lysis using RBC lysis buffer (BioLegend) and an additional enrichment step using magnetic bead separation for $CD66b^+$ cells, as described above for PMN-MDSCs.

For T-cell isolation, PBMCs were obtained from the low-density fraction of Ficoll density gradient sedimentation and separated with

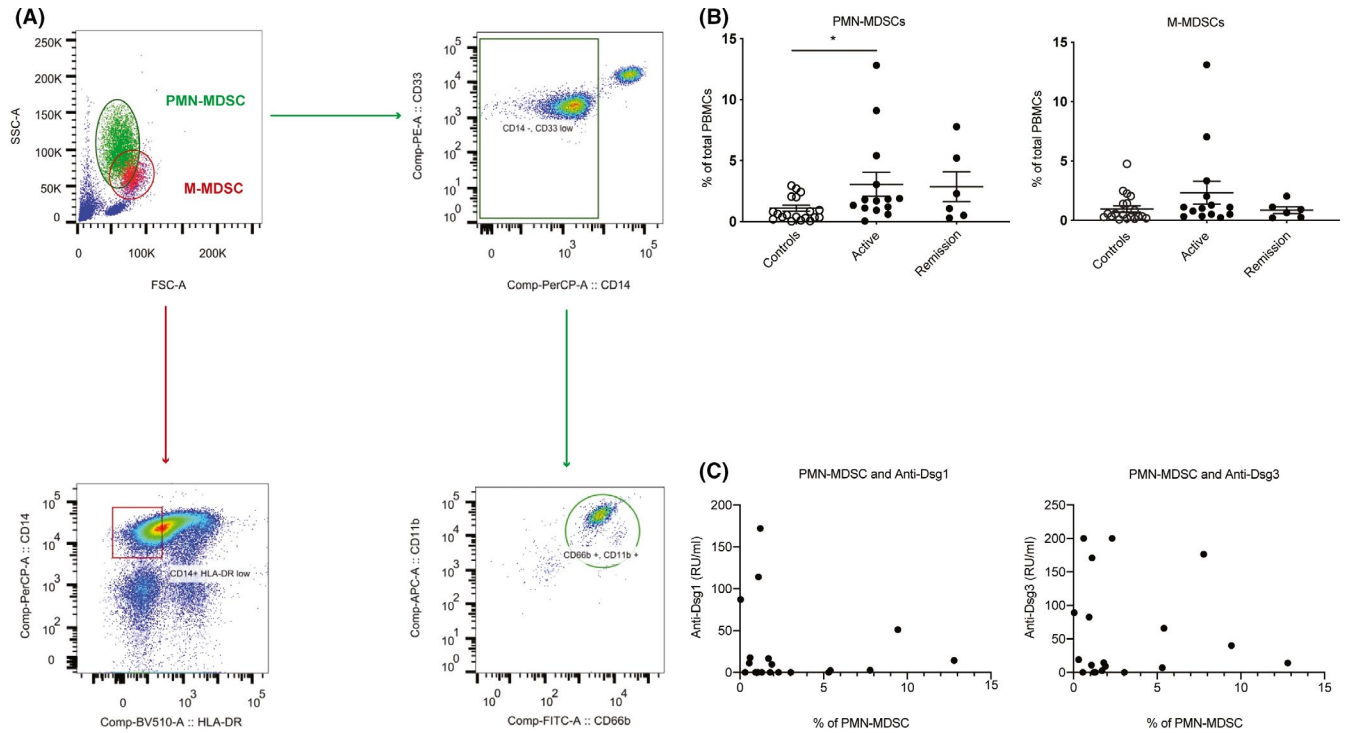

FIGURE 1 A, *Phenotypical characterization of MDSCs in Pemphigus*. Gating strategy for PMN-MDSCs and M-MDSCs. PBMCs from pemphigus patients were isolated via Ficoll density gradient. PMN-MDSCs were identified by $CD14^-$, $CD33^+$, $CD11b^+$ and $CD66b^+$. M-MDSCs were HLA-$DR^{-/low}$ and $CD14^+$. B, *Increased frequencies of PMN-MDSCs in Pemphigus patients*. Significantly increased frequencies of PMN-MDSCs, but not M-MDSCs, were found in the PBMC fraction of chronic active pemphigus patients compared with age-matched healthy controls and remissive patients. MDSCs frequencies are indicated as percentage of total PBMCs. Lines represent mean ± SEM. Controls $n = 19$, Patients $n = 20$. Statistical analysis was performed with Kruskal-Wallis test followed by Dunn's multiple comparisons test, $^*p < 0.05$. C, *Correlations between frequencies of PMN-MDSCs and Desmoglein Levels*. No correlation could be found between frequencies of PMN-MDSCs and levels of Anti-Dsg 1 and Anti-Dsg 3 antibodies in patients' serum. Correlations were described with Spearman's correlation coefficient *r*.

anti-CD3 microbeads followed by two sequential magnetic bead separation steps (Miltenyi Biotec), according to the manufacturer's protocol. The purity of $CD66b^+$ and $CD3^+$ cells after separation was >90% as assessed by flow cytometry.

Antibodies against CD3, CD4, CD11b, CD14, CD25, CD33, HLA-DR, CD45RA, CD117, CXCR3, CXCR5 and CCR6 were purchased from BioLegend. Quantifications were performed with BD FACSDiva ™ and FlowJo™ (Becton, Dickinson and Company) analysis software.

2.3 | Quantitative RT-PCR

PMN-MDSCs and PMNs were isolated from pemphigus patients by magnetic separation as described above. Total RNA from PMN-MDSCs and PMNs was isolated using PeqGOLD Micro RNA Kit (VWR). Reverse transcription into cDNA was performed with Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher). Relative gene expression was determined by quantitative RT-PCR using TaqMan probes (TIB MolBiol) and the Roche LightCycler480 system (Roche). Beta-actin (*ACTB*) and RNA polymerase II subunit A (*POLR2A*) were used as housekeeping genes. The control condition was set to 1 for normalization. The following primers sequences and probes were used:

ACTB: for AgCCTCgCCTTTgCCgA, rev CTggTgCCTggggCg probe 6FAM-CCgCCgCCCgTCCACACCCgCC—BBQ

POLR2A: for gTggAgATCTTCACggTgCT, rev gTgCggCTgCTTC CATAA probe 6FAM-TACCACgTCATCTCCTTTgATggCTCCTAT—BBQ

S100A9: for CTgCAAAATTTTCTCAAgAAggAg, rev gCTCAg CTgCTTgTCTgCAT probe 6FAM-CATAgAACACATCATggAgg ACCTggACAC—BBQ

ARG1: for CAgAAgAAATCTACAAAACAgggC, rev CTCCCg AgCAAgTCCg probe 6FAM-CTgCTgTgTTCACTgTTCgAgTTACT TCT—BBQ

NOS2:forgAAACgCACAAgCTggCrevCCATgATggTCACATTCTgCT probe 6FAM-TCgCTCTggAAAgACCAggCTgTC—BBQ

IL10: for gCTACggCgCTgTCATCgA rev AgATgCCTTTC TCTTggAgCTTA probe 6FAM-ACCTgCTCCACggCCTTgCT—BBQ

TGFB1: for CTCCTgTgACAgCAgggATAAC rev CAgTTCTTC TCCgTggAgCTgA probe 6FAM-AAgTggACATCAACgggTTCACT ACCg—BBQ

IL4RA: for CgTCTCCgACTACATgAgCATCT rev CCACAgg TCCAgTgTATAgTTATCC probe 6FAM-CCTCCgTTgTTCTCAgggATA CACgT—BBQ

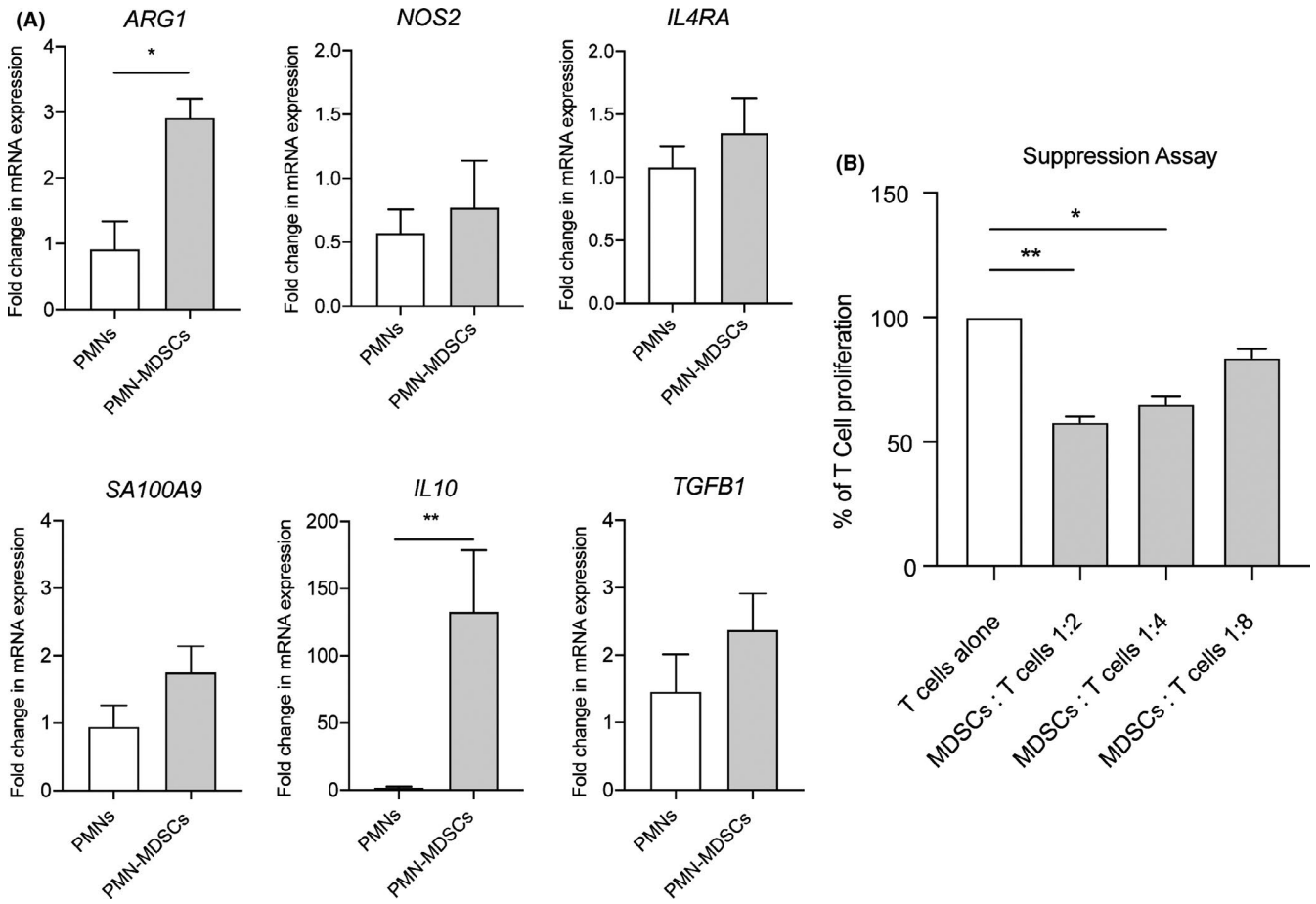

FIGURE 2 A, *Molecular characterization of PMN-MDSCs in Pemphigus*. After PBMC isolation through Ficoll density gradient, PMN-MDSCs were magnetically sorted using granulocytic marker CD66b. RT-PCR of MDSCs molecular hallmarks was performed with pemphigus patients PMN-MDSCs and compared with conventional PMNs from the same patients. Isolated PMN-MDSCs showed significantly increased expression of *ARG1* and *IL10*, which are classically associated with MDSCs' suppressive activity. Values are presented as mean of fold change in mRNA expression +SEM, $n = 3$ (*NOS2*), $n = 6$ (*TGFB1*), n = 7 (*SA100A9*) and $n = 8$ (*ARG1*, *IL4RA*, *IL10*). Statistical analysis was performed with Mann-Whitney test, $^{*}p < 0.05$, $^{**}p < 0.01$. B, *Functional characterization of PMN-MDSCs in Pemphigus*. Magnetically sorted PMN-MDSCs from pemphigus patients could effectively inhibit allogeneic T-cell proliferation after in vitro stimulation with Interleukin-2 and plate bound anti-CD3 and anti-CD28 antibodies. T-cell proliferation was assessed with 3H-Thymidine incorporation assay. MDSCs effect upon T-cell proliferation was concentration-dependent. Bars depict mean of % of T-cell proliferation +SEM, $n = 4$. Statistical analysis was performed with Friedman's test followed by Dunn's multiple comparisons test, $^{*}p < 0.05$, $^{***}p < 0.001$

2.4 | Suppression assay

PMN-MDSCs were isolated from pemphigus patients by magnetic separation as described above. Responder $CD3^{+}$ T cells were obtained by magnetic separation from healthy volunteers and were co-cultured with MDSCs at different reciprocal concentrations of 1:1, 2:1, 4:1 and 8:1 for 96 hours in RPMI 1640 medium (Merck KGaA) in a humidified atmosphere at 37°C and 5% CO_2. The cell culture was supplemented with 10% heat-inactivated human serum, 2 mM glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich). The number of T cells per well was kept at 10^5, and MDSCs were added accordingly. T-cell proliferation was stimulated with CD3/CD28-antibodies (5 µg/ml, Biolegend) and recombinant mouse interleukin-2 (IL-2) (100 U/ml, Biolegend). 3H-thymidine (PerkinElmer) was added to the cells at a final concentration of 1.25 mCi/ml for the last 24 h of incubation time, and incorporation was detected by a PerkinElmer 1450 Micro Beta TriLux Counter (PerkinElmer). Data were normalized to the stimulated T-cell proliferation control (without addition of PMN-MDSCs).

2.5 | Statistical analysis

Statistical analyses were performed using Prism 8.3.0. software (GraphPad Software). Differences between the control group and patients were calculated using Kruskal-Wallis test followed by Dunn's multiple comparisons test. Relative expression of PMN-MDSC biochemical parameters was related to expression in conventional PMNs and analysed with Mann-Whitney test. Differences within suppression assay were determined with Friedman's test followed by Dunn's multiple comparisons test. Correlations were described with Spearman's correlation coefficient *r*. Error bars

were depicted as mean +SEM. p-Values < 0.05 were considered statistically significant (with $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

3 | RESULTS

3.1 | A population of neutrophil-like myeloid cells is increased in pemphigus patients

To investigate whether MDSCs might be expanded in pemphigus, we first performed a cross-sectional analysis of 20 pemphigus patients (Table 1). We found that pemphigus patients exhibited a significantly increased population of neutrophil-like myeloid cells in the Ficoll low-density fraction of peripheral blood compared with age-matched healthy controls (Figure 1A, B). These myeloid cells were phenotypically characterized as $CD11b^{+}$, $CD14^{-}$, $CD33^{+}$ and $CD66b^{+}$ and showed granulocytic morphology. On the basis of their phenotypical characterization, we postulated that these cells represent PMN-MDSCs. We further identified a population of $CD11b^{+}$, $CD14^{+}$, $CD33^{+}$ and HLA-$DR^{-/low}$ monocytic-like myeloid cells; however, no significant differences were found between pemphigus patients and healthy controls regarding this cell population. In our study, chronic active pemphigus patients but not remissive patients showed increased frequencies of PMN-MDSCs compared to healthy controls. All patients were receiving systemic immunosuppressants at the time of sampling; however, frequencies of MDSCs were similar among the different therapy groups, suggesting that accumulation of neutrophil-like myeloid cells was unrelated to the background therapy (data not shown). We did not find any significant correlation between PMN-MDSCs frequencies and anti-Dsg 1 or anti-Dsg 3 levels (Figure 1C).

3.2 | Neutrophil-like myeloid cells from pemphigus patients express arginase I and interleukin 10

Acute inflammatory or infectious conditions activate conventional neutrophils, which, in turn, are relatively short-lived and mainly act anti-microbial. In contrast, chronic inflammation results in persistent stimulation of the myeloid compartment with less intense signals, leading to the formation of neutrophilic cells with immunoregulatory properties (PMN-MDSCs). Under these conditions, PMN-MDSCs produce high levels of arginase I and reactive nitrogen species, as well as immunosuppressive cytokines. These markers are used to distinguish PMN-MDSCs from conventional neutrophils. To further characterize the elevated myeloid cell population found in pemphigus patients, we performed RT-PCR of the aforementioned molecular factors associated with MDSC activity.[5] Compared to conventional PMNs, PMN-MDSCs showed significantly increased expression of *ARG1* and *IL10*, whereas no difference in the expression of *NOS2* or *IL4RA* could be detected (Figure 2A). Expression of iNOS and IL-4Rα is associated with the M-MDSCs phenotype, while ARG1 is increased in PMN-MDSCs.[37] These findings are in line with our surface receptor characterization of a neutrophil-like myeloid cells population in pemphigus patients and support the classification of these cells as PMN-MDSCs.

3.3 | Isolated PMN-MDSCs from pemphigus patients inhibit T-cell proliferation in vitro

To confirm, whether the increased myeloid cells detected in pemphigus belong also functionally to PMN-MDSCs, we sought to test their suppressive activity on T cells. We isolated MDSCs from pemphigus patients and performed co-cultures together with allogeneic responder T cells stimulated with anti-CD3/CD28 antibodies and IL-2 using different cell ratios. After 4 days, T-cell proliferation was assessed by 3H-Thymidine incorporation. MDSCs could efficiently suppress T-cell proliferation in a cell ratio-dependent manner (Figure 2B). These results confirm that the neutrophil-like myeloid cells in pemphigus patients express not only phenotypical PMN-MDSCs markers but also exhibit suppressive activity as functional PMN-MDSCs.

3.4 | PMN-MDSCs directly correlate with an increased Th2 response in active pemphigus

MDSCs exert not only a suppressive function on T-cell proliferation but have also been implied as regulators of the Th1/Th2 balance.[38] To investigate, whether MDSCs may influence T helper responses in pemphigus, we first compared the frequencies of different Th cell subsets between patients and healthy controls. Pemphigus patients in active disease stage showed significantly increased frequencies of Th2, Th17 and Tfh17 cells compared to healthy controls, whereas frequencies of Th1 and Tfh1 cells were decreased (Figure 3B). Pemphigus patients in remission showed lower frequencies of Tfreg cells compared to the healthy control group. To better dissect differences in the balance of Th cell subsets, we calculated the Th2/Th1, Tfh2/Tfh1, Treg/Th17 and Tfreg/Tfh17 ratios. When focusing on active pemphigus patients, we found increased Th2/Th1 and Tfh2/Tfh1 ratios, while Treg/Th17 and Tfreg/Tfh17 ratios were decreased (Figure 3C). Of note, PMN-MDSCs directly correlated with the Th2/Th1 cell ratios in patients with active disease (Figure 3D). These observations highlight an altered balance between Th cell subsets in pemphigus, which may reflect an interaction between MDSC and T cells.

4 | DISCUSSION

Our study provides evidence that a population of PMN-MDSCs is increased in pemphigus patients and can suppress T-cell proliferation in vitro. PMN-MDSCs were increased in active pemphigus patients, while no significant difference was found between the remission and healthy control groups. All patients in our cohort were receiving immunosuppressive therapy at the time of blood sampling, but no difference could be found among the different therapy groups, suggesting

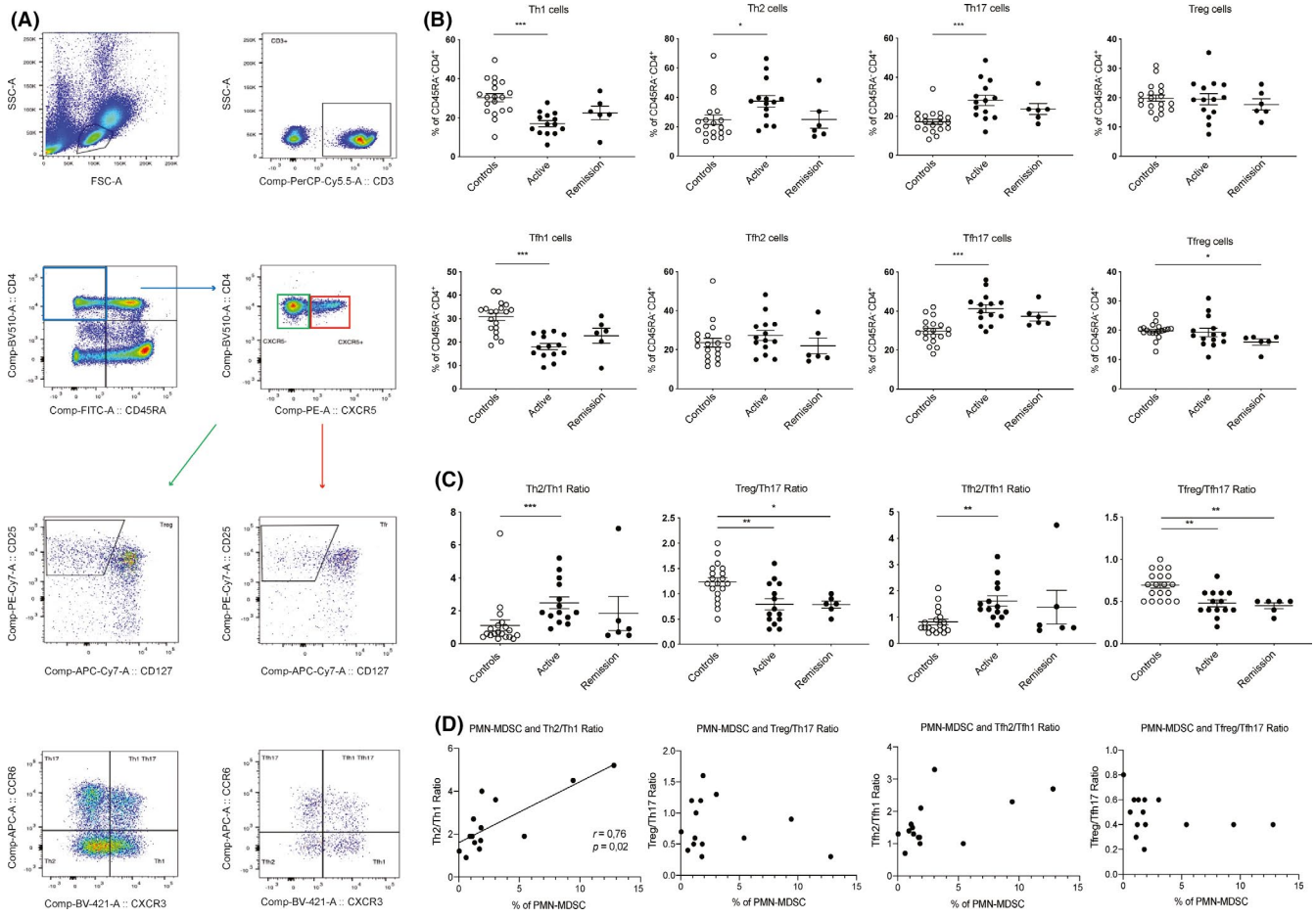

FIGURE 3 A, *Gating strategy for T-cell subsets*. Frequencies of different T helper (Th) and T follicular helper (Tfh) subsets were assessed by flow cytometry using specific chemokine receptors. Th cells and Tfh cells were distinguished upon CCR5 expression, T regulatory (Treg) and Tf regulatory (Tfreg) were distinguished upon expression of CD25 and CD127. B, *Differences in T helper and T follicular helper cell subsets between Pemphigus patients and healthy controls*. Chronic active Pemphigus patients showed increased frequencies of Th2, Th17 and Tfh17 cells and decreased Th1, Tfh1 and Tfreg cells. Frequencies of Th and Tfh subsets are indicated as percentages of $CD45RA^{-}$ $CD4^{+}$ cells. Lines represent mean ± SEM. Controls $n = 19$, Patients $n = 20$. Statistical analysis was performed with Kruskal-Wallis test followed by Dunn's multiple comparisons test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. C, *Differences in T helper and T follicular helper cell ratios between Pemphigus patients and healthy controls*. A statistically significant increase in Th2/Th1 and Tfh2/Tfh1 ratios as well as a decrease in Treg/Th17 and Tfreg/Tfh17 ratios suggest an alteration in Th and Tfh balance in active pemphigus. Remissive patients showed a statistically significant decrease in Treg/Th17 and Tfreg/Tfh17 ratios as well. However, no differences were found in Th2/Th1 or Tfh2/Tfh1 ratios. Statistical analysis was performed with Kruskal-Wallis test followed by Dunn's multiple comparisons test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. D, *Correlations between frequencies of PMN-MDSCs and different T helper cell ratios in chronic active Pemphigus patients*. PMN-MDSCs correlated significantly with increased frequencies of Th2 cells and decreased frequencies of Th1 cells, thus with an increased Th2/Th1 ratio in chronic active pemphigus patients. Correlations were described with Spearman's correlation coefficient *r*

that disease activity influences the expansion of MDSCs and not treatment regimens. However, we found no direct correlation between frequencies of MDSCs and Anti-Dsg 1/3 antibody levels, which have been proposed as markers of disease activity in pemphigus.[33]

In line with our findings, a very recent report described increased PMN-MDSCs in the blood of pemphigus vulgaris and bullous pemphigoid patients. The authors described increased levels of MDSCs in patients with active disease (either therapy naïve or therapy resistant), while patients responding to immunosuppressive therapy showed MDSCs frequencies comparable to healthy controls.[39]

The pathogenesis of Pemphigus has been the centre of several studies in the last decades, identifying autoreactive T cells as crucial initiators of the autoimmune response against desmogleins. In a humanized mouse model of pemphigus, Dsg 3-reactive $CD4^{+}$ T cells were implied in the activation of B cells and the resulting production of anti-Dsg 3 antibodies, which ultimately initiated acantholysis.[40] Serological data in humans support the role of Dsg 3-reactive Th2 cells in stimulating B-cell secretion of autoantibodies in PV.[25] Another population of interest is represented by the Tfh cells, which are increased in pemphigus patients and may thus play a role in the generation of autoantibodies by B cells.[26,41] Our characterization of Th cell and Tfh cell responses in pemphigus showed some differences in the frequency of subsets, especially in active patients. The alterations of the Th2/Th1 and Tfh2/Tfh1 balance as well as the

Treg/Th17 and Tfreg/Tfh17 balance seem to be relevant for pemphigus pathogenesis.[42] Since the discovery of MDSCs, the mutual relationship between Th cells and MDSCs has been subject of extensive investigations.[38] MDSCs have been implied not only in the suppression of T-cell responses, but also in the modulation of Th cell development. MDSCs have been reported to elicit antigen-specific T-cell tolerance by inducing expansion of FoxP3$^+$ Treg cells.[43] Accordingly, transfer of MDSCs into allogeneic bone marrow transplant recipient mice ameliorated graft-versus-host disease (GVHD) through Tregs induction.[44] Furthermore, MDSCs appear to regulate the developmental program of Th17 and Treg cells, acting at the crossroad of Treg/Th17 differentiation.[11] Interestingly, we found no correlation between MDSCs and Treg cells, whose frequencies were not altered in active pemphigus patients compared to healthy controls. Thus, it seems that the presence of MDSCs does not influence Treg development in the setting of pemphigus, as described for other autoimmune conditions.

MDSCs did not correlate with Treg cells or anti-Dsg autoantibody levels in our cohort. They also did not correlate with the ratio of Tfh cell populations, which are thought to be relevant for autoantibody production by B cells. In contrast, MDSCs did correlate with the Th2/Th1 ratio in active pemphigus patients, which is interesting in the context that increased Th2/Th1 ratios were found in active patients but not in the remission group. These data support the theory that the altered polarization of the Th2/Th1 balance might represent a MDSC-related factor in pemphigus pathogenesis.[25]

The relationship between MDSCs and Th2 cells has been documented in many reports, and it is conceivable that Th2 cells play a role in MDSC activation. Th2 lymphocytes are classically implied in defence against parasites such as helminthes. A study conducted on mice infected with the cestode *Taenia Crassiceps* described a strong Th2 response with high levels of the Th2 cytokines IL-4 and IL-13, which induced a CD11b$^+$ Gr-1$^+$ MDSCs population with increased arginase activity.[45] In addition, bone marrow MDSCs ameliorated GVHD in a murine model via an arginase-1-dependent mechanism.[46] In this study, a potent MDSC subset with suppressive function was obtained from stimulation with the exogenous Th2 cytokine IL-13. Intriguingly, another study on murine GVHD reported that in vitro–generated MDSCs prevent murine GVHD by inducing Th2 cells, which were indispensable for MDSC activation and GVHD prevention.[47]

In our study, PMN-MDSCs were increased in active but not in remissive patients, and only active patients showed an increased Th2/Th1 ratio compared to healthy controls. It is feasible to hypothesize that the expansion of a suppressive myeloid population in pemphigus is related to the altered balance of Th2/Th1 cells and may act as a countermeasure to the skewed Th2/Th1 responses in the context of chronic inflammation. However, further analyses are necessary to dissect, whether the above-mentioned relationship between Th2 cells and MDSCs is functional and essential in pemphigus pathogenesis.

Another interesting finding is represented by the expansion of Th17 and Tfh17 cells in pemphigus patients.[26] MDSCs have been implied in the generation of Th17 from naïve CD4$^+$ T cells.[48] In rheumatoid arthritis, one report linked MDSC expansion with heightened Th17 response and disease progression,[49] while another study showed how the adoptive transfer of MDSCs in a mouse model prevented the development of autoimmune arthritis through inhibition of Th17 cell development.[50] In experimental autoimmune encephalomyelitis (EAE), a disease classically associated with Th17 response, MDSCs were found to correlate with disease progression and Th17 cell induction.[51] However, also in this setting controversial reports exist. For example, MDSCs have been reported to exert a tolerogenic function and when transferred adoptively they ameliorated EAE symptoms in mice.[52] These reports suggest that the relationship between MDSCs and Th17 cells in the pathogenesis of autoimmune diseases is not univocal and may vary among the different diseases and experimental settings. In our study, Th17 cells and Tfh17 cells were increased in chronic active but not in remissive patients. However, no correlation was found with these phenotypes and MDSC frequencies. Yet, longitudinal studies with larger cohorts would help to further dissect the potential role of MDSC populations and Th/Tfh cell subsets in pemphigus patients.

Our study presents some limitations. The major limitation is the small size of the examined cohort, which is due to the rare nature of the studied disease. Moreover, the severity of the disease demands systemic immunosuppressive treatments as received by the patients studied here, which may impact MDSC frequencies and functionality. It is also important to note that while anti-Dsg antibodies have been suggested to correlate with disease activity in the literature, the levels of circulating antibodies can vary among patients as well as the extent of their clinical manifestations. Under immunosuppression, some of our patients did not show detectable levels of circulating anti-Dsg1 and/or anti-Dsg3 antibodies at the time of blood sampling but presented positive anti-Dsg1 and/or anti-Dsg3 antibodies at the time of diagnosis. A longitudinal study would be required to address whether MDSC frequencies reflect disease activity and autoantibody levels over time.

In conclusion, our study describes a population of suppressive myeloid cells in the autoimmune blistering skin disease pemphigus. These myeloid cells express granulocytic markers, exhibit high levels of *ARG1* and *IL10*, and are capable of suppressing T-cell proliferation, thus being consistent with the PMN-MDSCs phenotype. Pemphigus patients with active disease but not in remission showed an expanded population of PMN-MDSCs. PMN-MDSCs frequencies correlated with an increased ratio of Th2/Th1 cells in active pemphigus, supporting a link between skewed Th2/Th1 balance and accumulation of MDSCs in autoimmunity. Further studies are required to better characterize the influence of MDSCs on Th/Tfh cell differentiation in pemphigus and to provide new insights into the potential role of these cells in autoimmune blistering skin diseases.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

AUTHOR CONTRIBUTIONS

KG conceived and supervised the study. DN, MCN, JB, JH and IS designed and performed the experiments. KG, DN, MCN and JB analysed the data. KG, DN, MCN and FS wrote the manuscript. KG, DN, MCN, IS, FS, JH, JB, DH and RH discussed data and conceptualized questions.

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REFERENCES

1. Zea AH, Rodriguez PC, Atkins MB, et al. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. *Cancer Res*. 2005;65(8):3044-3048.
2. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol*. 2009;9(3):162-174.
3. Wang Y, Schafer CC, Hough KP, et al. Myeloid-derived suppressor cells impair B cell responses in lung cancer through IL-7 and STAT5. *J Immunol*. 2018;201(1):278-295.
4. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol*. 2012;12(4):253-268.
5. Bronte V, Brandau S, Chen SH, et al. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. *Nat Commun*. 2016;7:12150.
6. Youn JI, Collazo M, Shalova IN, Biswas SK, Gabrilovich DI. Characterization of the nature of granulocytic myeloid-derived suppressor cells in tumor-bearing mice. *J Leukoc Biol*. 2012;91(1):167-181.
7. Movahedi K, Guilliams M, Van den Bossche J, et al. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. *Blood*. 2008;111(8):4233-4244.
8. Rodriguez PC, Quiceno DG, Ochoa AC. L-arginine availability regulates T-lymphocyte cell-cycle progression. *Blood*. 2007;109(4):1568-1573.
9. Schmielau J, Finn OJ. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of t-cell function in advanced cancer patients. *Cancer Res*. 2001;61(12):4756-4760.
10. Huang B, Pan PY, Li Q, et al. Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. *Cancer Res*. 2006;66(2):1123-1131.
11. Hoechst B, Gamrekelashvili J, Manns MP, Greten TF, Korangy F. Plasticity of human Th17 cells and iTregs is orchestrated by different subsets of myeloid cells. *Blood*. 2011;117(24):6532-6541.
12. Lelis FJN, Jaufmann J, Singh A, et al. Myeloid-derived suppressor cells modulate B-cell responses. *Immunol Lett*. 2017;188:108-115.
13. Jaufmann J, Lelis FJN, Teschner AC, et al. Human monocytic myeloid-derived suppressor cells impair B-cell phenotype and function in vitro. *Eur J Immunol*. 2020;50(1):33-47.
14. Park MJ, Lee SH, Kim EK, et al. Myeloid-derived suppressor cells induce the expansion of regulatory B cells and ameliorate autoimmunity in the sanroque mouse model of systemic lupus erythematosus. *Arthritis Rheumatol*. 2016;68(11):2717-2727.
15. Wu H, Zhen Y, Ma Z, et al. Arginase-1-dependent promotion of TH17 differentiation and disease progression by MDSCs in systemic lupus erythematosus. *Sci. Transl. Med.*. 2016;8(331):331ra340.
16. Kurko J, Vida A, Glant TT, et al. Identification of myeloid-derived suppressor cells in the synovial fluid of patients with rheumatoid arthritis: a pilot study. *BMC Musculoskelet Disord*. 2014;15:281.
17. Guo C, Hu F, Yi H, et al. Myeloid-derived suppressor cells have a proinflammatory role in the pathogenesis of autoimmune arthritis. *Ann Rheum Dis*. 2016;75(1):278-285.
18. Schmidt E, Kasperkiewicz M, Joly P. Pemphigus. *Lancet*. 2019;394(10201):882-894.
19. Amagai M, Klaus-Kovtun V, Stanley JR. Autoantibodies against a novel epithelial cadherin in pemphigus vulgaris, a disease of cell adhesion. *Cell*. 1991;67(5):869-877.
20. Amagai M, Tsunoda K, Zillikens D, Nagai T, Nishikawa T. The clinical phenotype of pemphigus is defined by the anti-desmoglein autoantibody profile. *J Am Acad Dermatol*. 1999;40(2 Pt 1):167-170.
21. Mindorf S, Dettmann IM, Krüger S, et al. Routine detection of serum antidesmocollin autoantibodies is only useful in patients with atypical pemphigus. *Exp Dermatol*. 2017;26(12):1267-1270.
22. Lotti R, Atene CG, Marconi A, et al. Development of a desmocollin-3 active mouse model recapitulating human atypical pemphigus. *Front Immunol.*. 2019;10:1387.
23. Ishii N, Teye K, Fukuda S, et al. Anti-desmocollin autoantibodies in nonclassical pemphigus. *Br J Dermatol*. 2015;173(1):59-68.
24. Solimani F, Maglie R, Pollmann R, et al. Thymoma-associated paraneoplastic autoimmune multiorgan syndrome-from pemphigus to lichenoid dermatitis. *Front Immunol.*. 2019;10:1413.
25. Hertl M, Eming R, Veldman C. T cell control in autoimmune bullous skin disorders. *J Clin Invest*. 2006;116(5):1159-1166.
26. Holstein J, Solimani F, Baum C, et al. Immunophenotyping in pemphigus reveals a T(H)17/T(FH)17 cell-dominated immune response promoting desmoglein1/3-specific autoantibody production. *J Allergy Clin Immunol*. 2020;S0091–6749(20):31624-31629.
27. Rizzo C, Fotino M, Zhang Y, Chow S, Spizuoco A, Sinha AA. Direct characterization of human T cells in pemphigus vulgaris reveals elevated autoantigen-specific Th2 activity in association with active disease. *Clin Exp Dermatol*. 2005;30(5):535-540.
28. Eming R, Budinger L, Riechers R, et al. Frequency analysis of autoreactive T-helper 1 and 2 cells in bullous pemphigoid and pemphigus vulgaris by enzyme-linked immunospot assay. *Br J Dermatol*. 2000;143(6):1279-1282.
29. Veldman C, Höhne A, Dieckmann D, Schuler G, Hertl M. Type I regulatory T cells specific for desmoglein 3 are more frequently detected in healthy individuals than in patients with pemphigus vulgaris. *J Immunol*. 2004;172(10):6468-6475.
30. Yuan H, Zhou S, Liu Z, et al. Pivotal role of lesional and perilesional T/B lymphocytes in pemphigus pathogenesis. *J Invest Dermatol*. 2017;137(11):2362-2370.
31. Kim AR, Han D, Choi JY, et al. Targeting inducible costimulator expressed on CXCR5(+)PD-1(+) T(H) cells suppresses the progression of pemphigus vulgaris. *J Allergy Clin Immunol*. 2020;146(5):1070-1079.e8.
32. Stoll H, Ost M, Singh A, et al. Staphylococcal enterotoxins dose-dependently modulate the generation of myeloid-derived suppressor cells. *Front Cell Infect Microbiol*. 2018;8:321.
33. Schmidt E, Dahnrich C, Rosemann A, et al. Novel ELISA systems for antibodies to desmoglein 1 and 3: correlation of disease activity with serum autoantibody levels in individual pemphigus patients. *Exp Dermatol*. 2010;19(5):458-463.
34. Boulard C, Duvert Lehembre S, Picard-Dahan C, et al. Calculation of cut-off values based on the Autoimmune Bullous Skin Disorder Intensity Score (ABSIS) and Pemphigus Disease Area Index (PDAI) pemphigus scoring systems for defining moderate, significant and extensive types of pemphigus. *Br J Dermatol*. 2016;175(1):142-149.
35. Mahnke YD, Beddall MH, Roederer M. OMIP-017: human CD4(+) helper T-cell subsets including follicular helper cells. *Cytometry A*. 2013;83(5):439-440.
36. de St F, Groth B, Zhu E, Asad S, Lee L. Flow cytometric detection of human regulatory T cells. *Methods Mole Biol*. 2011;707:263-279.
37. Romano A, Parrinello NL, La Cava P, et al. PMN-MDSC and arginase are increased in myeloma and may contribute to resistance to therapy. *Expert Rev Mol Diagn*. 2018;18(7):675-683.

38. Nagaraj S, Youn J-I, Gabrilovich DI. Reciprocal relationship between myeloid-derived suppressor cells and T cells. *J Immunol*. 2013;191(1):17-23.
39. Oktem A, Horzum U, Ertop P, et al. Clinical relevance of polymorphonuclear myeloid-derived suppressor cells in autoimmune-blistering disorders pemphigus vulgaris and bullous pemphigoid. *J Invest Dermatol*. 2020.
40. Eming R, Hennerici T, Backlund J, et al. Pathogenic IgG antibodies against desmoglein 3 in pemphigus vulgaris are regulated by HLA-DRB1*04:02-restricted T cells. *J Immunol*. 2014;193(9):4391-4399.
41. Hennerici T, Pollmann R, Schmidt T, et al. Increased frequency of T follicular helper cells and elevated interleukin-27 plasma levels in patients with pemphigus. *PLoS One*. 2016;11(2):e0148919.
42. Meier K, Holstein J, Solimani F, Waschke J, Ghoreschi K. Case report: apremilast for therapy-resistant pemphigus vulgaris. *Front Immunol*.. 2020;11:588315.
43. Serafini P, Mgebroff S, Noonan K, Borrello I. Myeloid-derived suppressor cells promote cross-tolerance in B-cell lymphoma by expanding regulatory T cells. *Cancer Res*. 2008;68(13):5439-5449.
44. MacDonald KP, Rowe V, Clouston AD, et al. Cytokine expanded myeloid precursors function as regulatory antigen-presenting cells and promote tolerance through IL-10-producing regulatory T cells. *J Immunol*. 2005;174(4):1841-1850.
45. Brys L, Beschin A, Raes G, et al. Reactive oxygen species and 12/15-lipoxygenase contribute to the antiproliferative capacity of alternatively activated myeloid cells elicited during helminth infection. *J Immunol*. 2005;174(10):6095-6104.
46. Highfill SL, Rodriguez PC, Zhou Q, et al. Bone marrow myeloid-derived suppressor cells (MDSCs) inhibit graft-versus-host disease (GVHD) via an arginase-1-dependent mechanism that is up-regulated by interleukin-13. *Blood*. 2010;116(25):5738-5747.
47. Messmann JJ, Reisser T, Leithäuser F, Lutz MB, Debatin KM, Strauss G. In vitro-generated MDSCs prevent murine GVHD by inducing type 2 T cells without disabling antitumor cytotoxicity. *Blood*. 2015;126(9):1138-1148.
48. Chatterjee S, Das S, Chakraborty P, Manna A, Chatterjee M, Choudhuri SK. Myeloid derived suppressor cells (MDSCs) can induce the generation of Th17 response from naïve CD4+ T cells. *Immunobiology*. 2013;218(5):718-724.
49. Zhang H, Wang S, Huang Y, et al. Myeloid-derived suppressor cells are proinflammatory and regulate collagen-induced arthritis through manipulating Th17 cell differentiation. *Clin Immunol*. 2015;157(2):175-186.
50. Zhang L, Zhang Z, Zhang H, Wu M, Wang Y. Myeloid-derived suppressor cells protect mouse models from autoimmune arthritis via controlling inflammatory response. *Inflammation*. 2014;37(3):670-677.
51. Yi H, Guo C, Yu X, Zuo D, Wang XY. Mouse CD11b+Gr-1+ myeloid cells can promote Th17 cell differentiation and experimental autoimmune encephalomyelitis. *J Immunol*. 2012;189(9):4295-4304.
52. Casacuberta-Serra S, Costa C, Eixarch H, et al. Myeloid-derived suppressor cells expressing a self-antigen ameliorate experimental autoimmune encephalomyelitis. *Exp Neurol*. 2016;286:50-60.

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