Reduction of non-classical monocytes that suppress interferon-α in patients with systemic lupus erythematosus

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Abstract

Objective

Monocytes are known to be involved in both adaptive and innate immune responses, though their roles in the pathogenesis of systemic lupus erythematosus (SLE) are still unclear. Here, we performed phenotypic and functional analyses of each monocyte subset.

Methods

Peripheral blood from patients with autoimmune diseases (SLE: n=53, rheumatoid arthritis: n=12, systemic sclerosis: n=36) was analysed using flow cytometry to compare the number of each monocyte subset and the expression levels of the cell surface markers of patients to those of healthy donors (n=28).

Results

The number of $CD14^{dim}CD16^{+}$ non-classical monocytes in peripheral blood from SLE patients was significantly decreased compared with those from healthy donors and patients with other autoimmune diseases. The number of circulating non-classical monocytes was inversely correlated to SLE disease activity. The number of non-classical monocytes was not related to the use of glucocorticoids or to the presence or absence of specific tissue inflammation. The expression levels of cell surface molecules and the survival rate of non-classical monocytes of patients with SLE were similar to those of healthy donors. An in vitro functional assay revealed that non-classical monocytes suppressed IFN-α production from PBMCs or plasmacytoid DCs, and cell-cell contact through ICAM-4 seemed to be important in this process.

Conclusion

Our study demonstrated that the number of circulating non-classical monocytes, which has been shown to have the ability to suppress IFN-α production, was decreased in SLE patients, and this might be related to the excess IFN signature in SLE patients.

Key words

cells, immune system diseases, cell phenomena and immunity, immune system phenomena

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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterised by heterogeneity among the patients, who exhibit various types of autoantibodies and tissue damage. Treatments for SLE largely depend on the long-term administration of glucocorticoids and immunosuppressants, and novel drugs are desired due to the adverse effects and the risk of infection that are associated with conventional drugs (1, 2). Because it has been known that in SLE autoantibodies make immune complexes (ICs), which results in tissue damage like lupus nephritis (LN) as a result of renal deposition of ICs in SLE patients, lymphocytes such as autoantibody-producing B cells have been the focus of research for many years. Although innate immune cells have recently been shown to contribute to SLE pathogenesis, there has been relatively little research focused on monocytes.

Monocytes, accounting for 10% of peripheral blood mononuclear cells (PBMC), play an important role in innate immune responses. Although it has been thought that monocytes are precursor cells that migrate into inflammatory tissues and differentiate into macrophages and even into dendritic cells, recent studies have started to reveal the dynamics and functions of monocytes themselves. Nevertheless, the actual destinies and functions of monocytes *in vivo* remain largely unclear. For a long time, CD14 had been used as a marker for monocytes, but the existence of $CD16^+$ monocytes was subsequently discovered (3). Currently, it is known about the existence of three distinct human monocyte subsets, based on CD14 and CD16 expression levels: classical ($CD14^+CD16^-$), intermediate ($CD14^+CD16^+$) and non-classical ($CD14^{dim}CD16^+$) (4). Classical monocytes have been shown to migrate to sites of inflammation, and can differentiate into macrophages, so that they contribute to the promotion and/or resolution of the inflammation. Intermediate monocytes produce the highest levels of proinflammatory cytokines and have the highest expression levels of antigen presentation molecules, suggesting that this subset also plays a role in inflammatory diseases. Actually, increased levels of intermediate monocytes have been reported in diseases including sepsis (5), active Crohn's disease (6), and rheumatoid arthritis (RA) (7, 8). On the other hand, non-classical monocytes are called 'patrolling monocytes', having the role of checking condition of endothelial cells in the vasculature in a steady state (9, 10). However, the precise roles of these subsets *in vivo*, especially on diseases such as autoimmune diseases, remain unclear.

In the present study, we performed immunophenotyping of circulating monocyte subsets in patients with SLE and other autoimmune diseases to examine the involvement of monocytes in the pathogenesis of SLE. We also analysed the functions of the monocytes of healthy donors to predict their *in vivo* roles.

Materials and methods

Patients

The peripheral blood from patients with SLE (n=53), rheumatoid arthritis (RA) (n=12), or systemic sclerosis (SSc) (n=36), who exhibited disease activity as shown in Table I and required the initiation or intensification of treatment and were admitted to our hospital between November 2017 and March 2020, as well as from healthy individuals (n=28), was used in this study. The patients who were enrolled in this study were diagnosed according to the classification criteria for SLE (11-13) , RA (14) and SSc (15-17). The disease activity of SLE was assessed using the Safety of Estrogens in Lupus Erythematosus National Assessment Systemic Lupus Erythematosus Disease Activity Index (SELENA-SLEDAI) (18), and based on pathological analysis, the types of LN in patients with LN were as follows; I/II; n=3, III/III+V; n=12, IV/IV+V; n=10, V; n=1, N/A; n=3. The disease activity of RA and the skin sclerosis of SSc patients were assessed using the Simplified Disease Activity Index (SDAI) (19) and the modified Rodnan total skin thickness score (mRSS) (20), respectively. Blood samples were collected either before initial treatment or before treatment intensification during a flare-up to minimise the influence of treatment. The Human Ethics Re-

Table I. Demographic and clinical characteristics of the study groups.

	SLE (n=53)	RA (n=12)	SSc (n=36)	Healthy controls (n=28)
Age (years old)	41.5 (15-71)	69.6 (34-85)	65.1 (17-89)	34.8 (25-47)
Female subjects, n (%)	53 (100%)	7 (58.3%)	29 (80.6%)	16 (57.1%)
Disease duration (years)	11.7 (0-69.4)	1.0 (0.0-7.0)	6.3 (0.3-28.5)	
SELENA-SLEDAI score	8.2 (0-29)			
SDAI score		24.4 (9.3-55.1)		
m-Rodnan TSS score			11.9 (1-30)	
BILAG score, A1 or B2, n (%)	25 (47.2%)			
Anti-dsDNA antibody (U/mL)	40.5 (1.6->400)			
Manifestations, n (%)				
Renal	29 (54.7%)			
Neurological	12 (23.1%)			
Carditis	6 (11.5%)			
Arthritis	20 (38.5%)			
Cutaneous	22 (41.5%)			
Treatment, n (%)				
Hydroxcychloroquine	19 (35.8%)			
Immunosuppressants	22 (41.5%)	0 (0.0%)	9 (25.0%)	
Corticosteroids	37 (69.8%)	1 (8.3%)	0 (0.0%)	
(low to middle dose/ high dose)	(29/8)	(1/0)	(0/0)	
Biological DMARDs	1 1.9%)	0 (0.0%)	0 (0.0%)	
Methotrexate	2 (3.8%)	1 (8.3%)	0 (0.0%)	

The data shown are the number of patients or the mean of values, with the range or percentage in parentheses. High-dose corticosteroids: prednisone >30 mg/day. SDAI score: Simple Disease Activity Index; DMARDs: disease-modifying anti-rheumatic drugs.

view Committees of both the University of Occupational and Environmental Health and the Mitsubishi Tanabe Pharma Corporation reviewed and approved this study. Signed informed consent forms were obtained from all subjects prior to inclusion in this study.

Flow cytometric analysis

The whole blood of the SLE, RA, and SSc patients and the healthy subjects were stained with the fluorochrome-conjugated monoclonal antibodies described in Supplementary Table S1. After staining, Lyse/Fix Buffer (BD Biosciences) was used to haemolyse and fix the cells. The cells were analysed using FACSVerse and FlowJo software (BD Biosciences,). Isotype-matched control antibodies were used to evaluate backgrounds when calculating the ΔMFI for the expression levels of some surface molecules.

Cell preparation and culture

PBMCs were isolated from peripheral blood using Lympholyte-H Cell Separation Media (Cedarlane Laboratories), and cultured in RPMI1640 supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 U/mL streptomycin. In some experiments, CD14 microbeads, Pan-Monocyte isolation Kit and Diamond Plasmacytoid Dendritic Cell Isolation Kit II (Miltenyi Biotec) were used to separate or deplete $CD14^+$ monocytes, pan-monocytes and plasmacytoid dendritic cells (pDCs), respectively. For monocyte subset depletion experiment, isolated pan-monocyte was further separated into CD14 positive and negative cells using CD14 microbeads, and cocultured with pan-monocyte-depleted PBMCs. To isolate each monocyte subset, pan-monocytes isolated by magnetic cell separation were stained with anti-CD14 and CD16 antibodies, and cell sorting was performed using FACS Aria II (BD Biosciences). The purity of the isolated cells was always more than 90%.

Toll-like receptor (TLR) stimulation

PBMCs (1×10^5 cells/well, 96-well flat bottom plates) or pDCs (4×10^3 cells/well, 96-well round bottom plates) with or without monocytes were stimulated with the TLR9 agonist CpG ODN 2216 (0.5 μmol/L; InvivoGen) for 24 hours. In some experiments, neutralising antibodies (1 μg/mL) against ICAM-1, 2, 3 (BioLegend) and 4 (R&D Systems) were used to block the intermolecular interactions. In other experiment, isolated $CD14^+$ cells (1×10^5 cells/well in 96 well flat bottom plate) were stimulated with loxoribine (1 μmol/L), CpG ODN 2216 (0.5 μmol/L), poly I:C HMV (2 μg/mL), or imiquimod (2 μg/mL; InvivoGen, San Diego, CA, USA) for 24 hours.

Measurement of IFN-α

The IFN-α concentration in the culture supernatant was measured using Human IFN-α Flex Set, FACSVerse/Lyric, and FCAP Array software (BD Biosciences). To evaluate the IFN-α concentration in plasma, S-PLEX Human IFN-α2a Kit and MESO QuickPlex SQ 120 (Meso Scale Diagnostics) were used.

Quantitative real-time PCR

Total RNA was extracted using the RNAeasy Mini Kit (Qiagen). First-strand cDNA was synthesised using the High Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific). Quantitative real-time PCR was performed using THUNDERBIRD SYBR qPCR Mix (TOYOBO) and the StepOne Plus system (Thermo Fisher Scientific). Quantification was performed using a comparative CT method employing glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. The primer sequences used in this study are described in Supplementary Table S2.

Flow cytometric analysis for apoptotic and dead monocytes

Each monocyte subset was stimulated with or without 1 μM loxoribine or 0.5 μM CpG ODN2216 for 3 hours in the culture medium. PBMCs were isolated and stained with Propidium Iodide (PI) (BD Biosciences) and APC-conjugated Annexin V (BioLegend) in the Annexin Binding Buffer (BioLegend) and analysed using FACSVerse and FlowJo software (BD Biosciences).

Statistical analysis

Differences were examined using Student's *t*-test, and a correlation analysis was performed using Spearman's correlation test calculated by Graph Pad Prism software (GraphPad Software). A *p*-value of less than 0.05 was considered statistically significant.

Results

The number of circulating non-classical monocytes was decreased in patients with SLE

To analyse the circulating monocyte subsets in patients with autoimmune diseases, we performed immunophenotyping of the monocytes in peripheral blood using flow cytometry. The characteristics of participating SLE, RA, and SSc patients and healthy donors in this study are shown in Table I, and the monocyte gating strategy is shown in Figure 1A. Pan-monocytes were divided into three subsets depending on CD14 and CD16; expression levels: classical ($CD14^{+}CD16^{-}$), intermediate ($CD14^{+}CD16^{+}$), and non-classical ($CD14^{dim}CD16^{+}$). The numbers of pan-monocytes and each monocyte subset in the peripheral blood of patients with SLE, RA, and SSc tended to be similar to or higher than those of healthy donors, except for non-classical monocytes decreased in SLE patients (Fig. 1B-C and Suppl. Fig. S1).

Next, we investigated the correlation between a reduction in circulating non-classical monocytes and the SLE disease activity. The numbers of classical and intermediate monocytes did not appear to be correlated with SELENA-SLEDAI (Fig. 1D). On the other hand, the number of non-classical monocytes was negatively correlated with the SELENA-SLEDAI scores (Fig. 1D), suggesting that severe SLE patients have fewer non-classical monocytes in circulation. We then measured the monocyte counts before and 6 months after treatment in SLE patients who visited our hospital at 6-month interval and had responded to therapy, whose SELENA-SLEDAI scores reduced more than 10 (the mean from 20.67±3.34 to 2.17±4.71). The treatments patients received are shown in Supplementary Table S3. The number of non-classical monocytes was notably increased at 6 months after treatment initiation (Fig. 1E). Although the numbers of classical and intermediate monocytes were also increased, the magnitude of the change in the non-classical monocytes was larger than those in the classical and intermediate monocytes (Fig. 1E). Taken together, we found that the number of circulating non-classical monocytes in patients with SLE was particularly lower than those in healthy donors and other autoimmune patients, and were negatively correlated with disease activity.

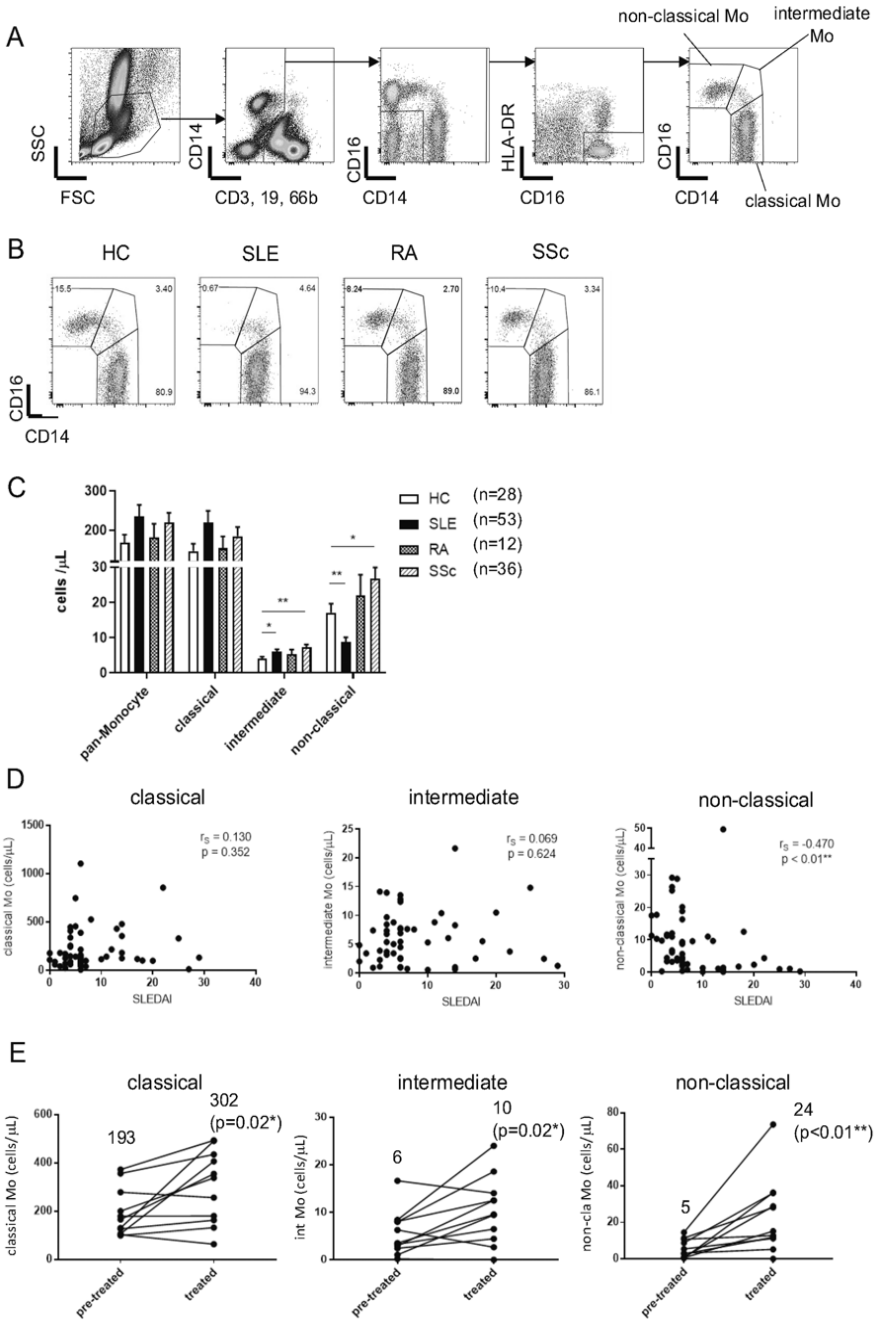

Fig. 1. Non-classical monocytes were decreased in patients with SLE. Whole blood was analysed using flow cytometry.
A: Gating strategy for monocyte subset identification.
B: Representative plots of monocytes.
C: The numbers of monocytes in indicated groups. Bars represent the mean+SEM.
D: Correlation between the number of each monocyte subset in SLE patients and SELENA-SLEDAI (SLEDAI).
E: Changes in the number of non-classical monocytes in SLE patients treated for 6 months (n=11). The floating numbers in graphs represent the mean values. Student's t-test (C, E) or Spearman's correlation test (D) were performed.
*p<0.05, **p<0.01. Mo: monocyte; HC: healthy donor control.

The number of circulating non-classical monocyte was not related to glucocorticoid treatment or specific types of tissue damage

To predict the reason for the small

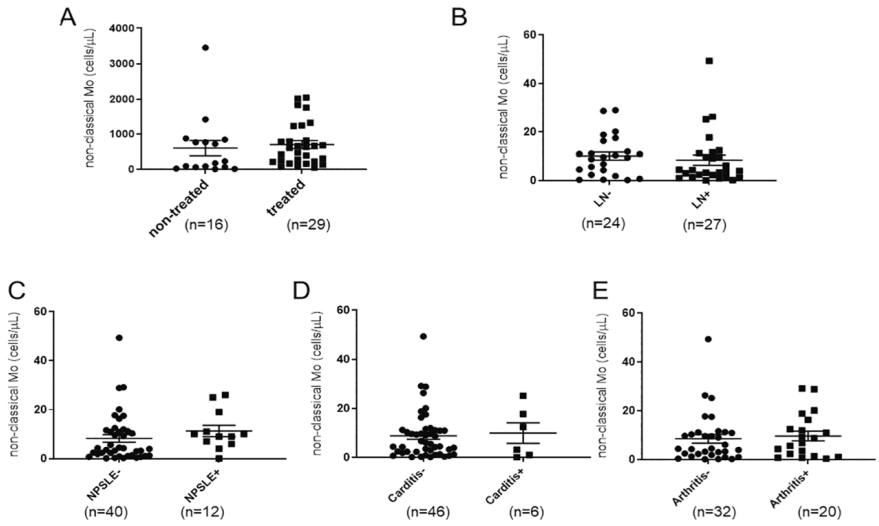

Fig. 2. The number of non-classical monocytes was not related to glucocorticoid treatment or tissue damage. Whole blood from patients with SLE was analysed using flow cytometry, and the number of non-classical monocytes with or without glucocorticoid treatment (**A**) in patients with or without lupus nephritis (LN) (**B**), neuropsychiatric (NP) SLE (**C**), carditis (**D**), and arthritis (**E**) were shown.
Horizontal lines represent the mean value of each group with SEM. There was no significant difference between the two groups (A-E) (Student's t-test).

number of non-classical monocytes in patients with SLE, we next investigated the relationships between the number of non-classical monocytes and glucocorticoid treatment or some symptoms of SLE. The number of non-classical monocyte was no different in patients with or without glucocorticoid treatment (Fig. 2A). Moreover, it was found that the number of circulating non-classical monocytes was not affected by the existence of specific types of tissue damage in SLE such as LN, neuropsychiatric SLE, carditis, or arthritis (Fig. 2B-E). In the end, we could not prove that the migration to inflamed tissues was the cause of the reduction in the number of circulating non-classical monocytes in patients with SLE.

Characteristics of non-classical monocytes were not substantially different in patients with SLE than in healthy donors

Next, we investigated the possibility that the characteristics of non-classical monocytes in patients with SLE were different from those in heathy donors. First, we analysed the expression levels of cell surface molecules related to

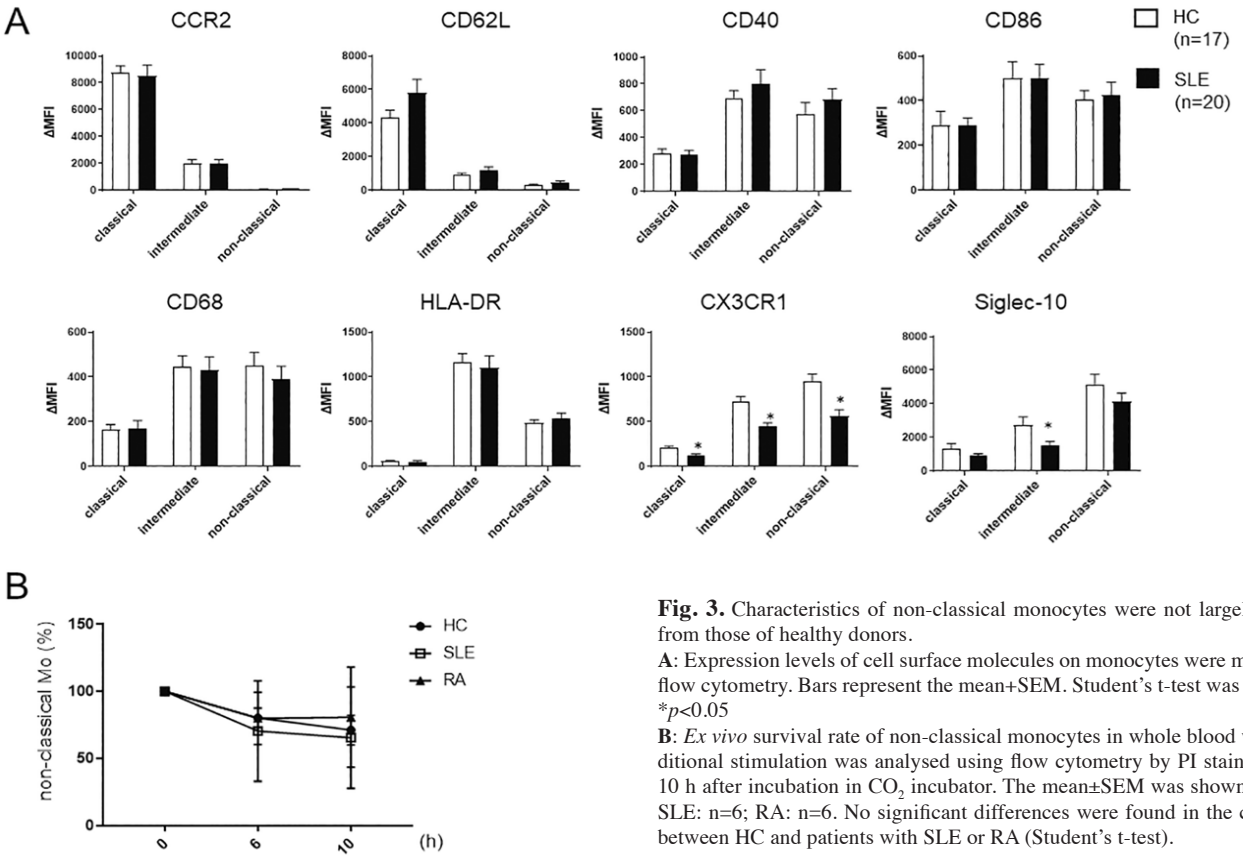

Fig. 3. Characteristics of non-classical monocytes were not largely different from those of healthy donors.
A: Expression levels of cell surface molecules on monocytes were measured by flow cytometry. Bars represent the mean+SEM. Student's t-test was performed. **p*<0.05
B: *Ex vivo* survival rate of non-classical monocytes in whole blood without additional stimulation was analysed using flow cytometry by PI staining at 0, 6, 10 h after incubation in CO_2 incubator. The mean±SEM was shown. HC: n=6; SLE: n=6; RA: n=6. No significant differences were found in the comparison between HC and patients with SLE or RA (Student's t-test).

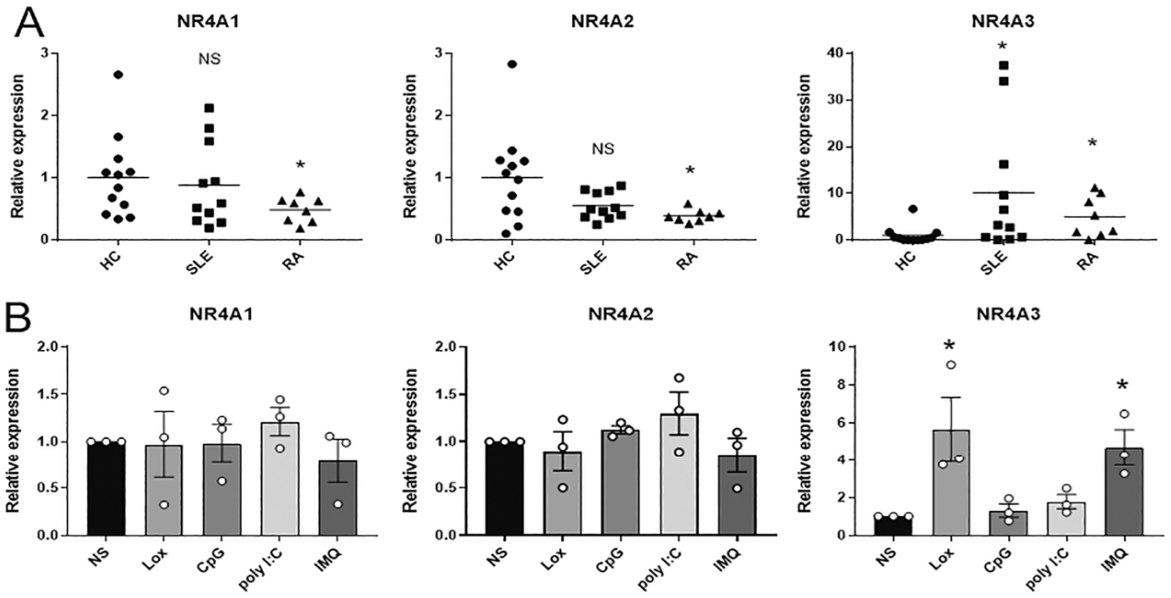

Fig. 4. CD14⁺ monocytes of healthy donors changed to SLE-like phenotype through TLR7 signalling. The expression levels of NR4A on CD14⁺ monocytes from HC, SLE and RA (**A**) or HC (**B**) were measured using qPCR. Values are normalised to GAPDH. **A**: Bars represent the mean of each group. Student's t-test (*vs*. HC) was performed. **p*<0.05.
B: The expression levels were measured after 24 hours stimulation with indicated TLR agonists. NS: no stimulation; Lox: loxoribine; IMQ: imiquimod. Bars represent the mean+S.E.M. of 3 healthy donors and data are representative of two independent experiments. **p*<0.05, by student's paired t-test (*vs*. NS).

cell migration, antigen presentation, and activation, in addition to the distinctive molecules of non-classical monocytes. The expression levels of the cell surface molecules examined in this study in each monocyte subset, including non-classical monocytes in SLE patients, were not largely different from those in healthy donors, although CX3CR1 expression was lower in SLE monocytes (Fig. 3A). Then we compared the *ex vivo* survival rate of non-classical monocytes from SLE patients to that of non-classical monocytes from healthy donors. Over a fixed period, the non-classical monocyte survival rates in blood in SLE patients, healthy donors, and RA patients were similar (Fig. 3B). In addition, we examined whether TLR7 and 9 pathways, which were thought to contribute to the pathogenesis of SLE, induced non-classical monocyte cell death. When monocytes from healthy donors were stimulated with agonists for these receptors, cell deaths induced by the TLR7 agonist loxoribine tended to be increased, but subset specific effects were not observed (Suppl. Fig. S2).

Taken together, there seemed to be no big differences between the non-classical monocytes of SLE patients and those of healthy donors in terms of cell surface molecule expression and the *ex vivo* survival rate, and there was no evidence of differences in the characteristics of non-classical monocytes resulting in the reduction in the number of these cells in patients with SLE.

NR4A3 expression on CD14⁺ monocytes was upregulated in SLE patients

We next considered the possibility that a signal distinctive to SLE patients had been transduced into the precursors of non-classical monocytes, resulting in an effect on the differentiation of these cells. Recent studies have shown that non-classical monocytes are differentiated from classical monocytes through intermediate monocytes (21). In mice, it has been reported that the transcription factor NR4A1 is essential for the differentiation of Ly6C⁻ monocytes (equivalent to non-classical in humans) (22). Therefore, we isolated CD14⁺ monocytes (classical and intermediate) from patients with SLE and examined their NR4A expression levels using quantitative real-time PCR. The NR4A1 and 2 expression levels in the CD14⁺ monocytes from SLE patients were similar to that in those from healthy donors, but the NR4A3 expression level was higher (Fig. 4A). Furthermore, when CD14⁺ monocytes from healthy donors were stimulated with TLR7 agonists, the NR4A3 expression levels were increased (Fig. 4B). In addition to NR4A, we compared the expression levels of TNFR2, which might be involved in the differentiation of non-classical monocytes (23), on the cell surface of monocytes in patients with SLE to those in healthy donors. However, no differences were observed between SLE patients and healthy donors, at least in the TNFR2 expression levels in each monocyte subset (Suppl. Fig. S3).

From these data, it was suggested that the nature of CD14⁺ monocytes, which have been considered to be the precursor cells of non-classical monocytes, are different in SLE patients and healthy donors, and this may affect their differentiation into non-classical monocytes.

Non-classical monocytes suppressed IFN-α production by PBMCs

Because the number of circulating non-classical monocytes in SLE patients decreased as disease activity increased, we hypothesised that the lack of non-classical monocytes might affect the features of SLE patients, such as a high IFN signature. To clarify this point, we next examined the role of non-classical monocytes on IFN-α production from PBMCs, including pDCs. Monocyte

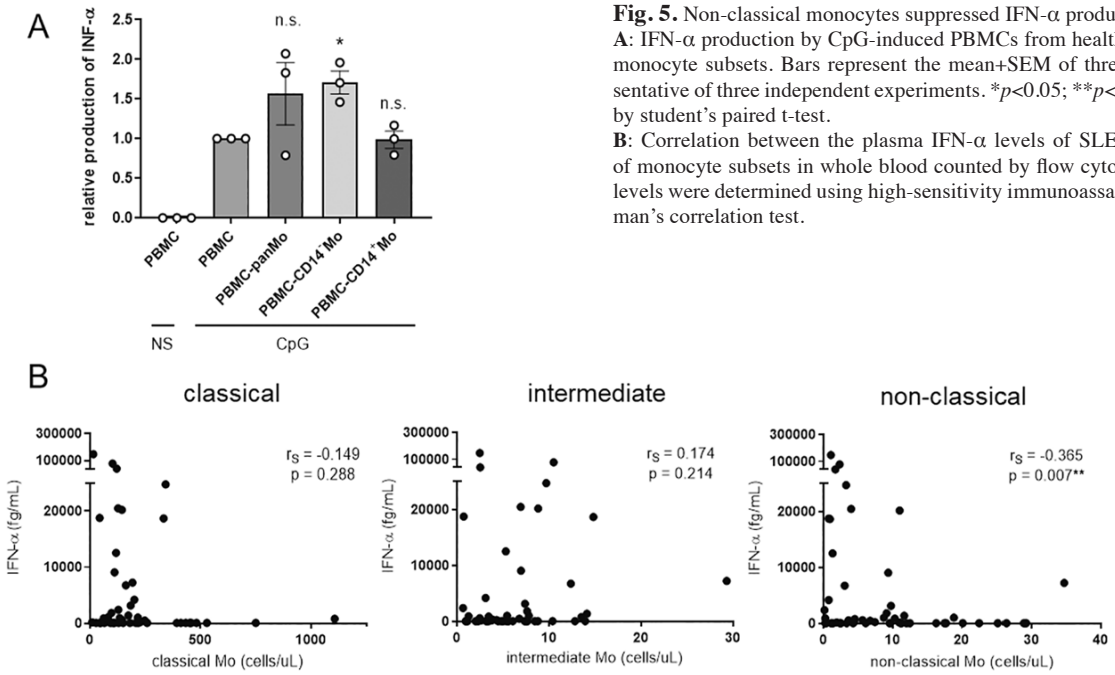

Fig. 5. Non-classical monocytes suppressed IFN-α production by PBMCs.
A: IFN-α production by CpG-induced PBMCs from healthy donors with or without monocyte subsets. Bars represent the mean+SEM of three donors. Data are representative of three independent experiments. *p<0.05; **p<0.01, compared to PBMC, by student's paired t-test.
B: Correlation between the plasma IFN-α levels of SLE patients and the number of monocyte subsets in whole blood counted by flow cytometry. The plasma IFN-α levels were determined using high-sensitivity immunoassay. **p<0.01, by the Spearman's correlation test.

subsets in PBMCs from healthy donors were depleted by magnetic cell separation system to compare the amount of IFN-α in the culture supernatant with or without each monocyte subset, with minimal effect on the original ratio of each cell population in the PBMCs. When $CD14^-$ (non-classical) monocytes were depleted, CpG-induced IFN-α production from PBMCs was enhanced, whereas depletion of $CD14^+$ classical and intermediate monocytes had no effect on IFN-α production (Fig. 5A), suggesting that non-classical monocytes act to suppress INF-α production. Moreover, the number of non-classical monocytes was negatively correlated to the IFN-α concentration in the plasma of SLE patients, while no correlations were found for classical and intermediate monocytes (Fig. 5B).

Taken together, our findings suggest that non-classical monocytes suppress IFN-α production by PBMCs, and the reduction in the number of non-classical monocytes in SLE patients might play a role in the high IFN-α production that is observed in SLE patients.

Non-humoral factor is essential for the suppression of IFN-α production by PBMCs

Although we elucidated the role of non-classical monocytes in suppressing IFN-α production, the way of suppression was not clear. In order to reveal the contribution of humoral factors such as cytokines, we used transwell to separate each monocyte subset from other cells in PBMCs. When we separated $CD14^+$ monocytes from other cells by transwell, the CpG-induced IFN-α production by PBMCs was not different from that which was seen without any separation (Fig. 6A). On the other hand, IFN-α production by PBMCs was slightly enhanced by $CD14^-$ monocyte separation, although not in a statistically significant manner (Fig. 6A). Since it was well known that pDCs produce considerable IFN-α and might contribute to SLE pathogenesis, we next separated pDCs and non-classical monocytes using magnetic beads and a cell sorter, and used coculturing to examine the direct effects of non-classical monocytes on pDCs in terms of IFN-α production. Coculturing of non-classical monocytes with pDCs resulted in lower concentrations of IFN-α in the culture supernatants compared to pDCs only, suggesting that non-classical monocytes directly suppressed IFN-α production by pDCs (Fig. 6B). Intermediate monocytes also suppressed IFN-α production by pDCs, but the effect was weaker than that of non-classical monocytes, and may have been caused by the contamination of non-classical monocytes due to the difficulty of isolating monocyte subsets cleanly.

Finally, to explore the cell surface molecules that are responsible for suppressing IFN-α production, we focused on ICAM-4, which is reported to be highly expressed on non-classical monocytes, and we tried inhibiting cell-cell contact through ICAM-4 using anti-ICAM-4 neutralising antibody (24). First, the expression levels of ICAM-4 on each monocyte subset were analysed and, consistent with the report by Wong *et al.*, the expression levels of ICAM-4 on non-classical monocytes were found to be higher than those on other monocyte subsets (Fig. 6C). Treatment with anti-ICAM-4 antibody enhanced IFN-α production by PBMCs (Fig. 6D), while treatment with anti-ICAM-1, 2, 3 antibodies did not have any effect (Suppl. Fig. S4).

Taken together, these findings suggest that cell-cell contact through ICAM-4 plays at least some roles in the suppressive effect of non-classical monocytes on IFN-α production by PBMCs.

Discussion

In the present study, we found that circulating non-classical monocytes were decreased in patients with SLE compared to healthy donors and patients with other autoimmune diseases. The possible reasons for the reduction are as follows: i) the migration into inflammatory tissues; ii) cell death specifically

of non-classical monocytes; iii) defects in differentiation into non-classical monocytes. Several previous studies have shown that non-classical monocytes might migrate and infiltrate into the kidneys of patients with LN (25-27). However, at least in our results, no relationship was observed between the number of circulating non-classical monocytes and the existence of specific tissue damage such as LN, and we could not obtain any evidence that this migration is the direct cause of the reduction in circulating non-classical monocytes. In addition to tissue migration, cell-specific death or abnormal differentiation into non-classical monocytes were considered possible factors behind the reduction in circulating non-classical monocytes (28). However, based on our results, it seems unlikely that non-classical monocyte-specific cell death was induced. Regarding the possibility of abnormal differentiation, on the other hand, when we analysed CD14$^+$ monocytes, which are said to be the precursors of non-classical monocytes, we found that CD14$^+$ monocytes from patients with SLE expressed higher levels of NR4A3 than those from healthy donors. Although little is known about the involvement of NR4A3 in the differentiation to non-classical monocytes, recent studies have shown that NR4A3 contributes to the differentiation of monocytes into dendritic cells (DCs) and macrophages. It has been shown that the expression levels of NR4A3 are increased when monocytes differentiate into DCs, and that NR4A3 expression is lower in the Ly6C$^-$ monocytes in mice than in Ly6C$^+$ monocytes (22, 29). Moreover, Gamrekelashvili *et al*. has reported that stimulation of TLR7 promotes the differentiation of mouse Ly6C$^+$ monocytes into macrophages and/or DCs instead of into Ly6C$^-$ monocytes (30). These reports suggest that the upregulation of NR4A3 in SLE monocytes caused CD14$^+$ monocytes to differentiate into DCs and/or macrophages more than into non-classical monocytes (likely through TLR7 signalling). In other words, it was thought that monocytes from SLE patients are in a state where they are more likely to differentiate into macrophages and DCs due to excessive stimulation of TLR7, and this may be a cause of the reduction in non-classical monocytes in peripheral blood. Additional analysis is needed to prove this hypothesis.

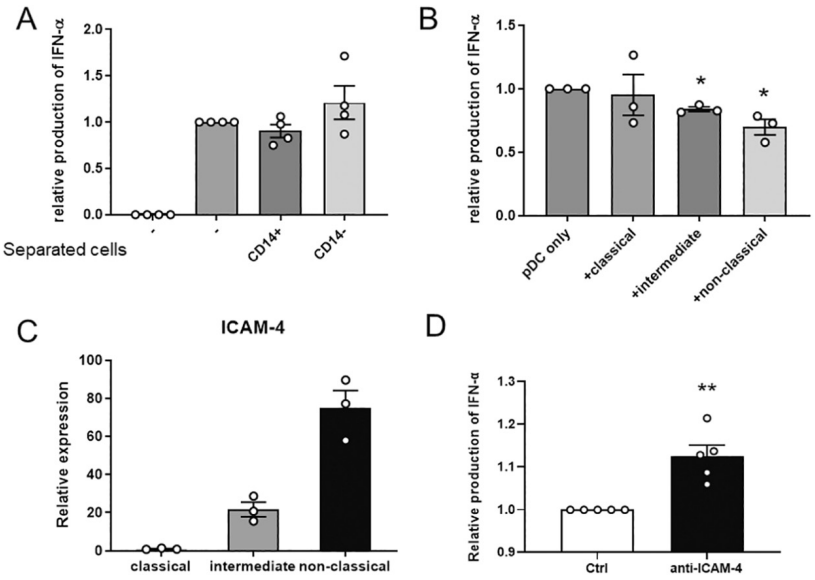

Fig. 6. Non-humoral factors contribute to the suppressive effect of non-classical monocytes on IFN-α production. (**A**, **B**, **D**) CpG-induced production of IFN-α by PBMCs (**A**, **D**) or pDC ±monocytes (**B**) from healthy donors. Indicated monocyte subsets were separated using transwell from other cells in PBMCs (**A**). pDC was cocultured with monocyte subset (**B**). Anti-ICAM-4 antibody was added to PBMCs on CpG-stimulation (**D**). (**C**) Expression levels of ICAM-4 in each monocyte subset. Bars represent the mean+S.E.M.. Data are representative of two or three independent experiments. *p<0.05, (*vs*. pDC only) (B), **p<0.01 (*vs*. Ctrl) (**D**) by Student's paired t-test.

In addition to flow cytometric analysis using blood of patients, we performed functional analysis of non-classical monocytes from healthy donors to clarify the influence of the reduction of circulating non-classical monocytes in patients with SLE. As a result, we demonstrated a novel function of non-classical monocytes to suppress the production of IFN-α by PBMCs (pDCs). We also showed that there was an inverse correlation between the concentration of IFN-α in the plasma from patients with SLE and the number of circulating non-classical monocytes, suggesting that the reduction in the number of non-classical monocytes may result in an enhanced IFN signature in SLE. Moreover, our findings show that cell-cell contact might be important for the suppressive effect of non-classical monocytes on IFN-α production by PBMCs. Wong et al. recently performed microarray analysis using each monocyte subset to clarify the subset-specific molecules (24), one of which, ICAM-4, became the subject of our focus. It was well known that ICAM-4 is expressed on erythrocytes, and it is thought to contribute to phagocytosis through CD11a/b/c expressed on macrophages (31, 32). Our experiments demonstrated that anti-ICAM-4 antibody treatment enhanced IFN-α production by PBMCs, suggesting that the ICAM-4 that is expressed on non-classical monocytes might contribute to the suppression of INF-α production. However, further analysis is required to prove this, and to identify the molecules on pDCs (or other cells such as monocytes) that interact with ICAM-4. Other challenges are to prove the interaction between pDCs and non-classical monocytes and to clarify where non-classical monocytes contact pDCs. As recent studies have revealed the importance of cell-cell interactions in small blood vessels, it seems possible that non-classical monocytes interact with pDCs in the blood, as well as in inflamed local tissues.

Since non-classical monocytes from patients with SLE are extremely rare, there are ethical problems with conducting mechanistic analysis, so several issues still remain. However, our findings of the reduction of non-classical monocytes in patients with SLE demonstrated that investigating non-clas-

sical monocytes and associated molecules may lead to the identification of biomarkers which can help to confirm SLE disease activity. In addition, we demonstrated for the first time that one function of non-classical monocytes is to suppress INF-α, thereby contributing to the understanding not only of SLE pathogenesis but also of the functions of monocyte subsets. Monocytes may be involved in the flare-up of autoimmune diseases such as RA, making them attractive targets for the development of new drugs target for autoimmune diseases (33).

Further research may contribute to the discovery of novel drugs targeting monocytes.

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