Journal of Clinical & Cellular Immunology

Liu et al., J Clin Cell Immunol 2019, 10:1
DOI: 10.4172/2155-9899.1000575

Research Article Open Access

Gene Expression Analysis on Neutrophil-Dendritic Hybrid Cells in Synovial Fluid from Rheumatoid Arthritis (RA) Patient

Yun Liu[1], Toshio Ota[2], Xiaohuan Chen[1], Hallie Dolin[1], Karen Pan[1], Akira Takashima[1], Atsushi Hinohara[2] and Zhixing K Pan[1*]

[1]*Department of Medical Microbiology and Immunology, The University of Toledo Health Science Campus, Toledo, OH, USA*

[2]*Alliance Management Group, Open Innovation Dept., R&D Division, Kyowa Hakko Kirin Co.,Ltd. 1188 Shimotogari, Nagaizumi-cho, Sunto-gun, Shizuoka, Japan*

[*]**Corresponding author:** Zhixing K Pan, Department of Medical Microbiology and Immunology, The University of Toledo Health Science Campus, Toledo, OH-43614 USA, Tel: +1 6468247052; E-mail: kevin.pan@utoledo.edu

Received date: February 14, 2019; **Accepted date:** February 22, 2019; **Published date:** February 28, 2019

Abstract

Neutrophils, as we have recently discovered, can differentiate into a unique leukocyte population expressing surface markers of both neutrophils and dendritic cells (DCs). The resulting population, termed "neutrophil-DC hybrid", retains the functionality of professional phagocytes while acquiring the properties of professional antigen presenting cells (APCs). In all tested mouse models of inflammatory diseases, hybrid cells emerged at inflammatory lesions, where they functioned as both phagocytes and APCs. Our central hypothesis is that neutrophil-DC hybrids play an important pathogenic role in a variety of inflammatory diseases, including rheumatoid arthritis (RA), psoriasis, and inflammatory bowel disease. If this is correct, one should then be able to treat these disorders by intercepting local transdifferentiation of neutrophils into the hybrids, blocking specific functions of the hybrid cells, or selectively killing the hybrid cells. As the first step in testing this concept, we are in the process of identifying unique genes that are expressed predominantly by the neutrophil-DC hybrids purified from synovial fluid samples from RA patients. We demonstrated that neutrophil-DC hybrids are present at the inflammatory lesions of RA patients. By using micro-array analysis, we investigated the gene expression profile of hybrids and the unique gene products expressed by neutrophil-DC hybrids in synovial fluid from rheumatoid arthritis patients. Our results demonstrated that transdifferentiation occurs in the joints of the RA patients, suggesting an expanded role for neutrophils in potential contribution to host adaptive immune processes.

Keywords: Rheumatoid arthritis; Neutrophil-dendritic hybrid cells; Gene expression

Introduction

Neutrophils constitute one of the first-responder populations arriving at sites of microbial infection. They have been thought to function primarily as phagocytes, conserved from ancient gene expression when terminally differentiated. However, there is emerging evidence that neutrophils can participate in adaptive immune responses by various mechanisms as well. Once activated after leaving the bone marrow, neutrophils undergo a transcriptional burst following rapid release of many signal mediators, such as granule contents [1], cytokines, chemokines, lipids [2], and reactive oxygen species (ROS) [3]. Those molecules are critical in downstream inflammatory processes and signaling cascades. In addition, the molecules launch the adaptive immune response by mobilizing and activating antigen-presenting cells (APCs), including monocytes, macrophages, and DCs. More directly, neutrophils can recruit T cells to inflamed tissues by chemokine-dependent reciprocal cross-talk [4], and promote immunoglobulin class-switching and antibody production by B cells [5].

Furthermore, in 1997, neutrophils were reported to acquire characteristics of typical professional APCs upon cultivation with interferon-gamma (IFN-γ) and granulocyte/macrophage colony-stimulating factor (GM-CSF) [6]. Many researchers have demonstrated that surface expression of DC markers, such as MHC II, CD86, CD83, and CCR6, became detectable in human neutrophils upon exposure to GM-CSF, IFN-γ, interleukin-3 (IL-3), and/or tumor necrosis factor-alpha (TNF-α) [7]. We also reported transdifferentiation of "neutrophil-DC hybrids" in neutrophils purified from mouse bone marrow (BM) in the presence of GM-CSF [8] and from the peritoneal cavity, skin, lung, and lymph nodes under inflammatory conditions [9]. These "neutrophil-DC hybrids" not only acquired DC markers and APC function, but also played dual immune protective roles. In accordance with our *in vivo* findings in mice, MHC II, CD80 and CD86 expression was observed in a significant fraction of peripheral blood neutrophils in patients with Wegener's granulomatosis [10], while few if any DC markers were expressed on the neutrophils of healthy control individuals [11,12]. Similarly, CD83 was found to be expressed in neutrophils from patients with acute bacterial infection [13], and elevated surface expression of CD40, CD64 and CD86 were reported on circulating neutrophils in sepsis patients [10]. Accumulation of a newly-discovered subset of tumor-associated neutrophils (TANs) which possess characteristics of both neutrophils and APCs were reported in early-stage human lung cancer. These hybrid neutrophils were described as able to trigger and augment anti-tumor T-cell responses through cross-presentation of antigens [14].

In the pathology of rheumatoid arthritis (RA), where infiltrating neutrophils play a key role, inflammatory neutrophils expressing cell surface receptors (such as MHC II and FcγRI or CD83) were enriched in the freshly isolated or cultured synovial fluid (SF) [15,16]. These alterations may be induced by T cell-derived cytokines [17], which can stimulate T cell proliferation in reverse. The above observations imply that transdifferentiation of band neutrophils into hybrid cells takes place in various types of inflammatory lesions, including those present in RA. Although recent research has raised scientific awareness of

neutrophil hybrids' existence and mechanisms, the transdifferentiation-associated changes occurring at the transcriptional level in the pathogenesis of RA remain unknown. Here, we demonstrated that neutrophil-DC hybrids are present at the inflammatory lesions of RA patients. We further employed RNA microarray analysis on DCs, neutrophils, and hybrid cells isolated from the synovial fluid (SF) of rheumatoid arthritis patients. By analyzing gene-expression data of the mouse bone marrow (BM)-derived neutrophil-DC hybrids, traditional DCs, and neutrophils from our previous publications, we characterized the transcriptional divergence of hybrids from experimental BM culture and clinical pathology lesions.

Materials and Methods

Patients and donors

After approval by the Institutional Review Board (IRB) of the University of Toledo and acquisition of informed consent, patients with rheumatoid arthritis visiting the Department of Rheumatology at the University of Toledo Medical Center were recruited in this study. A total of twelve patients were included. Each patient underwent a joint aspiration procedure as part of standard medical care to relieve RA-induced swelling and pain. The joint cavity was punctured and synovial fluid was collected in 50 mL tubes containing heparin and DNase. The fluid was then processed within 2 h.

Synovial fluid cell harvest

To reduce viscosity, samples were diluted with DPBS before filtration through nylon mesh. After centrifuge, the resulting pellet was incubated with Ca^{2+} and Mg^{2+} free DPBS containing 10 μg/ml DNase (Roche, USA), and 10 mg/ml hyaluronidase (Type IV, Sigma, USA) at 37°C for 15 min. To avoid residual erythrocyte contamination, the sample was lysed at room temperature.

Antibodies

For FACS analysis and sorting, anti-human monoclonal antibodies conjugated with specific fluorophores were purchased from R&D Systems and BioLegend, including APC-CEACAM-8/CD66b (Clone 913542), FITC-HLA-DR (Clone L243), PE- CD11c (Clone 3.9), PE-CD40 (Clone 5C3), PE-CD80 (Clone 2D10), PE-CD83 (Clone HB15e), and PE-CD86 (Clone IT2.2,).

Cell purification

CD66b was used as a neutrophil-specific marker, and CD86 and HLA-DR were chosen as the DC markers for sorting. Three cell populations were sorted using FACSAria II (BD Biosciences): CD66b +/ HLA-DR$^-$/CD86$^-$ conventional neutrophils, CD66b$^-$/HLA-DR+/CD86+ conventional DCs, and CD66b+/ HLA-DR+/CD86+ neutrophil-DC hybrids.

Cytospin and HE staining MPO

The sorted cells were processed with a CytospinTM 4 cytocentrifuge (Thermo Scientific, USA) at 2,000 rpm for 5 minutes. The samples were further treated with hematoxylin and eosin (H&E) staining using the HEMA 3 stain set (Protocol, USA).

RNA isolation

Total RNA was isolated from the sorted cells using TRIzol (Life Technologies) and RNAeasy columns (Qiagen, USA). After ethanol precipitation, the RNA was dissolved in RNase-free water and tested for quality control with a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA) and RNA 6000 Pico Kit (Agilent, USA).

RNA microarray

Samples were analyzed in the arrayAgilent Human SurePrint G3 GE Array. The data were imported into GeneSpring, and the differentially-expressed genes (DEGs) were screened among the three cell types. In each microarray, the data were normalized by linear interpolation with 75th percentile value, following the baseline transformation with the median of conventional DCs and neutrophils, and transformed to log values based "2".

Results

Phenotype assays of SF from RA patients show a variety of cell phenotypes

In total, we collected SF samples from twelve RA patients. The sample volumes varied substantially from 5 to 30 mL. These samples often showed a slightly yellow color and different degrees of turbidity. We prepared single-cell suspensions after enzymatic digestion of tissues. Most synovial fluid samples (8 out of 12) had relatively high cell yield (ranging from 6.00 10^6 to 2.46 10^8 cells), thus enabling FACS analyses (Table 1). At first, we tested various surface makers for neutrophils (CD66b) and for DCs (HLA-DR, CD11c, CD40, CD80, CD83 and CD86) in RA synovial fluid samples. We detected a large neutrophil population that stable and highly expressed CD66b in all tested samples (Figure 1A, representative sample). In order to improve the specificity for DC staining, we sought to distinguish the DC population with HLA- DR and other markers, namely CD86, CD80, CD40, and CD11c. As shown in Figure 1B, clear HLA-DR/CD40 and HLA-DR/86 double-positive populations appeared in CD66 negative population (the right panels), indicating that the DCs from SF highly expressed MHC class II antigens, CD40, and CD86. The expression of CD40 and/or CD86 in CD66 positive populations was also confirmed in almost all tested samples (7/8), except the sample stained with CD11c in our beginning protocol. Representative phenotype profiles within hybrid cells are shown (Figure 1C). These results further indicate the high specificity and consistency of the triple-staining strategy (CD66b/HLA-DR/CD86) for human SF samples.

Neutrophil-DC hybrids are quantifiably present in the synovial fluid of RA patients

Based on the phenotype data, we selected CD66b as a neutrophil marker and HLA-DR combined with CD86 as DC markers. Neutrophils, defined as CD66b+ cells without expressed DC markers were found to comprise the major leukocyte population in the synovial fluid samples, representing 18.5 to 74.0% of the total cell population (Figure 2A). DCs, defined as CD66b$^-$/HLA- DR+/CD86+ cells, were harvested in varying numbers, ranging from 3.9 to 46.8% of the total cell population (Figure 2A). Most importantly, neutrophil-DC hybrids, defined as CD66b+/HLA- DR+/CD86+ cells, were detectable in all clinical samples, albeit at relatively low-frequency (from 0.1 to 1.5%) (Figure 2A). In the scatter plot of FCS *vs.* SSC, although the

distribution of hybrids (in black) were adjacent to the neutrophils (in orange) due to shared characteristic granularity, with regard to size, the hybrids were more comparable with DCs (in green) (Figure 2B). To explore the morphology of hybrids, the three populations were spun down and stained with H&E. Most of the neutrophils contained typical segmented nuclei, but the hybrids showed some DC-like morphology characterized by oval-shaped nuclei, enlarged cell size, and dendritic processes (Figure 2C). Taken together, consistent with our previous research on equivalent cells in mice [8], the neutrophil-DC hybrids were indistinguishable from traditional DCs in morphology and expressed surface markers for both neutrophils and DCs. These observations validated our hypothesis that neutrophil-DC hybrids are present within inflammatory lesions in RA patients.

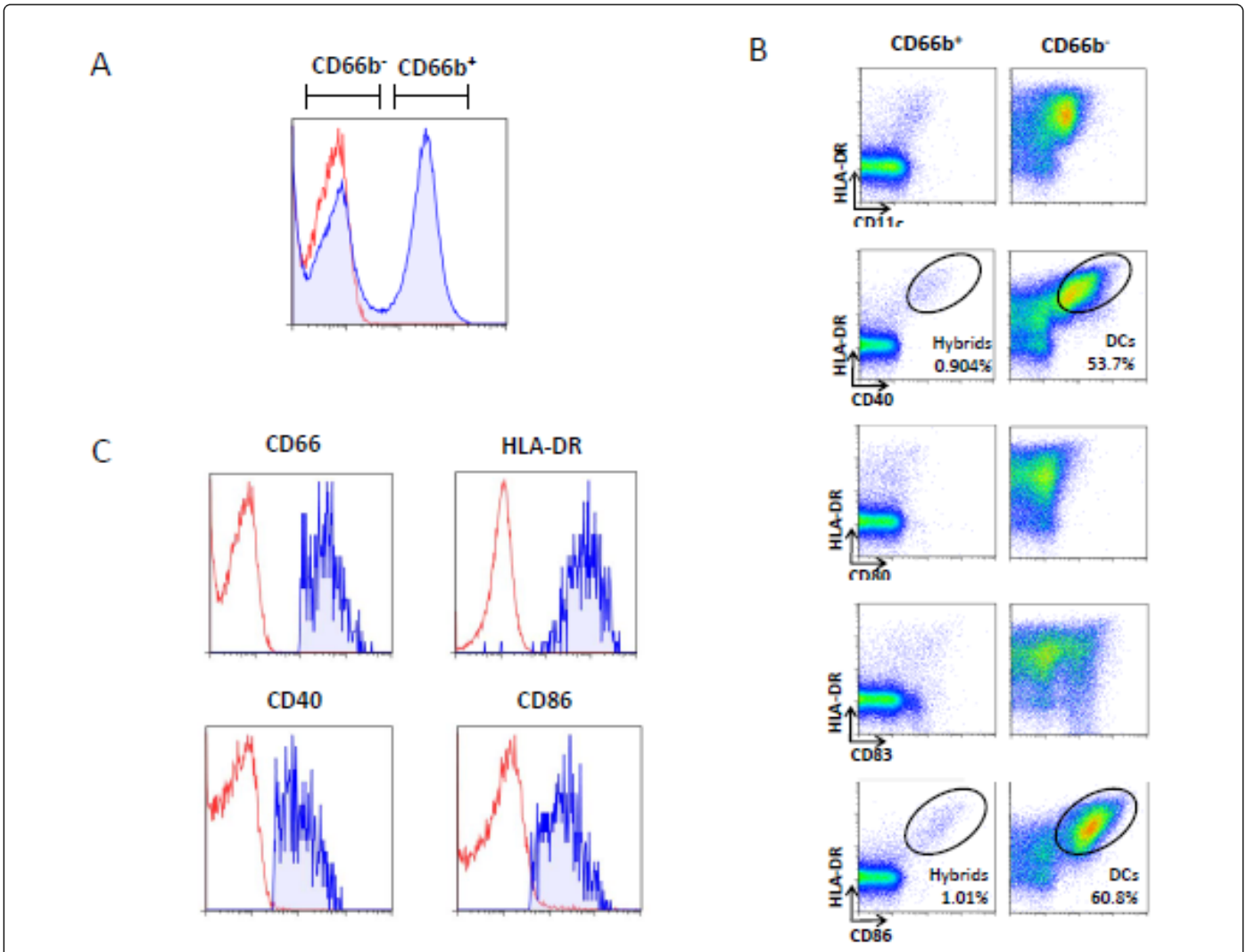

Figure 1: Surface marker expression of CD66b, HLA-DR, CD11c, CD40, CD86, and CD86 in synovial fluid (SF) from rheumatoid arthritis (RA) patient.

Gene expression profiles of neutrophil-DC hybrids resemble those of DCs more closely than neutrophils in synovial fluid of RA patients

To study the trans differentiation-associated changes at the mRNA level, we carried out gene expression analysis using RNA microarray on the limited hybrid cells from clinical SF samples. Fourteen samples were included in the microarray, including 5 samples from neutrophil populations, 5 from DC populations, and 4 from hybrid populations. By comparing signal intensity, we found a distribution of upregulated and downregulated genes, more than 1-fold higher than baseline in certain populations (Figure 3A). As we will address later, many DEGs in hybrid cells overlapped those in conventional DCs, but not those in neutrophils with respect to both up- and downregulated DEGs (Figure 3A). These findings imply that the hybrid cells are more similar to traditional DCs than neutrophils, but not identical with regards to gene expression patterns. 2,417 DEGs which represented those genes upregulated or downregulated more than 1- fold in DCs or neutrophils were gathered by 2-way clustering to assess the individual gene expression levels. Hybrid cells were clustered closely to traditional DCs, but were not identical, which was consistent with the above findings (Figure 3B). Our results suggested that the neutrophil-DC hybrids collected from human synovial fluid in RA resembled

traditional DCs not only in terms of morphology but also in gene expression profiles.

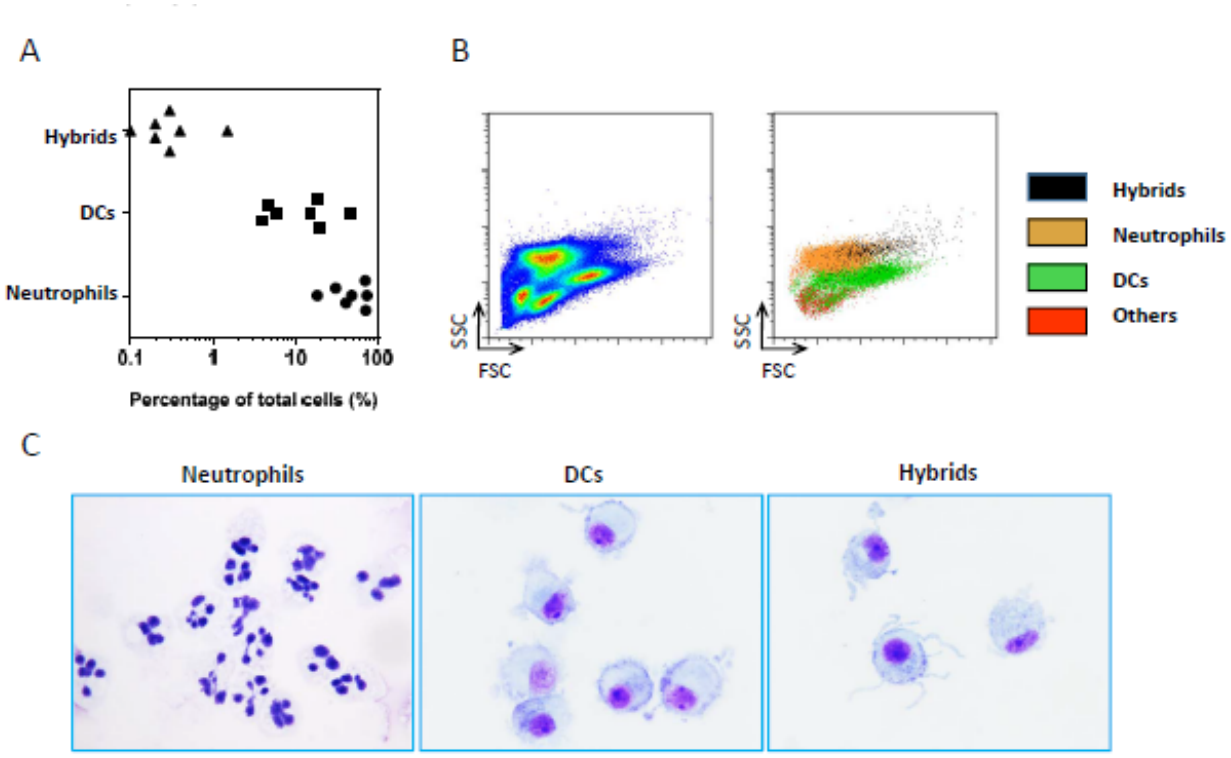

Figure 2: Emergence of neutrophils-DC hybrids in synovial fluid (SF) from rheumatoid arthritis (RA) patient.

ID of patients	Volume of synovial fluid sample (mL)	Total number of recovered cells (10^6)
1	22	20
2	15	6
3	30	100
4	17	0.4
5	17	140
6	5	30
7	20	130
8	25	2
9	15	140
10	15	246
11	5	1.1
12	12	3.1

Table 1: Recovery of synovial fluid samples.

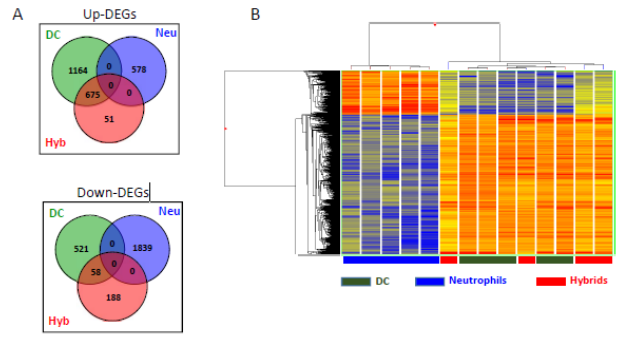

Figure 3: Gene-expression profiles of neutrophils, DCs and hybrids in synovial fluid (SF) from rheumatoid arthritis (RA) patient.

Expression levels of DC and neutrophil signature markers in three cell populations within synovial fluid show significant marker overlap

In order to explore the markers of neutrophil-DC hybrids, we next compared the expression of several surface molecules in the three populations, mainly the signature markers of neutrophils and DCs. For

example, CD66b, which is also known as carcinoembryonic antigen-related cell adhesion molecule 8 (CEACAM8), was highly expressed in neutrophils, but expressed at a lower level in hybrid cells (Figure 4). Strikingly, CD15, which plays a role in mediating phagocytosis, bactericidal activity, and chemotaxis and is present on >95% of granulocytes, did not show significant difference among the three cell populations (Figure 4). DCs and neutrophil-DC hybrids collected from human synovial fluid in RA both expressed the DC marker MHC II (HLA-DRA, HLADRB1, HLA-DRB3, HLA-DRB4, and HLA-DRB5), and co-stimulatory molecules (CD40, CD80, and CD86) at comparable levels. However, CD86 was also expressed by neutrophils in some of the RA cases (Figure 4). These observations suggested that neutrophil-DC hybrids were much closer to traditional DCs than to neutrophils in surface phenotype, which was consistent with the results from our gene expression analysis. Meanwhile, with intensity and variation of value taken into consideration, CD40 seemed to be a more specific DC-surface marker in distinguishing the dendritic cells within SF in RA patients, compared with CD80 and CD86. Although it is unknown if the hybrids express CD40 at high levels at the sites of other inflammatory lesions, this result may contribute to the optimization and standardization of staining protocol for hybrids isolated from RA patients in the future.

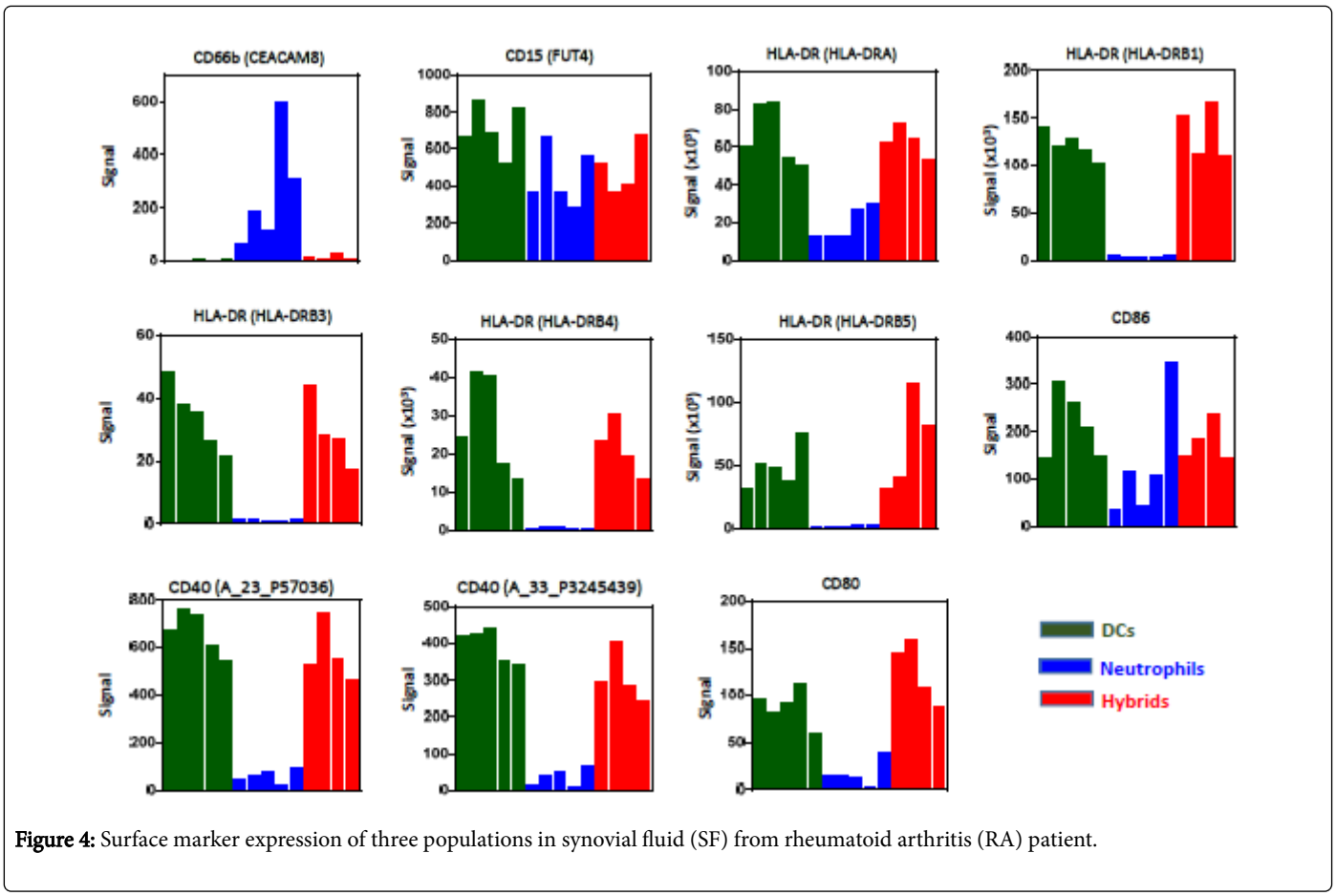

Figure 4: Surface marker expression of three populations in synovial fluid (SF) from rheumatoid arthritis (RA) patient.

Differentially-expressed genes in various cell populations are consistent in cells derived from mouse or human tissue

To investigate the differential gene expression in the three populations from mouse BM cells and synovial fluid in RA patients, we combined the previous published GeneChip data from Series GSE28408 [8], which was employed to compare the gene expression profiles between CD11c+/MHCII+/Ly6G+ DC (neutrophil-DC hybrids) and CD11c+/MHCII+/Ly6C$^-$ DC (conventional DC) with Series GSE53826 [18], which represented global gene expression profiles of neutrophils from BM Gr-1 high/CD48−neutrophils, together for re-analysis. That is to say, in the design of re-analysis, database on mice neutrophils sorted from Gr-1high/CD48$^-$, hybrid cells from CD11c+/MHCII+/Ly6G+, and DCs from CD11c+/MHCII +/Ly6G$^-$ were extracted for comparison with three populations in synovial fluid from RA patients. To begin with, among the dominated genes in mouse-derived neutrophils, MMP9, CD62L (SELL), CXCR2 (IL8RA), CRAMP (CAMP in human), CD45, GM-CSFR, and G-CSFR showed similar patterns in human synovial fluid samples. These genes were highly expressed in all neutrophil populations, but relatively low in DCs and hybrid cell populations, with the exception that CD24 was not always highly expressed in SF cell populations from RA patients. More importantly, consistent with results in mouse BM cells, hybrid cells from RA samples produced higher levels of CXCR2, CAMP, and G-CSFR, all of which are known to be abundant in neutrophils, than conventional DCs. In particular, CXCR2 (CXC receptor2) which appears to be neutrophils specific marker, and CAMP (cathelicidin antimicrobial peptide), which mediates bacterial killing in neutrophils, cannot be observed in DCs, but was observed in most hybrids (Figure 5A).

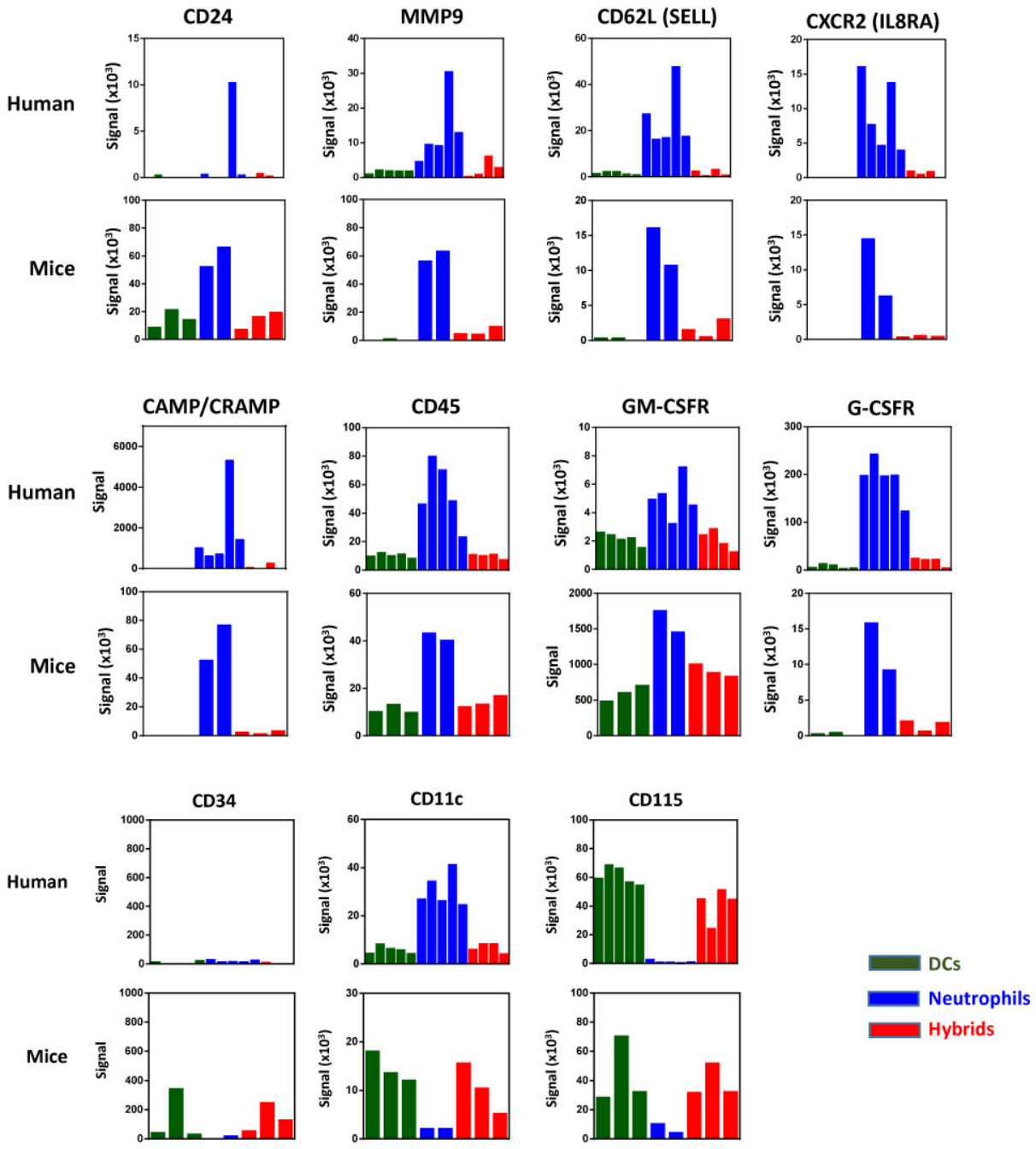

Figure 5: Published dominant genes expression three populations in mice and synovial fluid (SF) from rheumatoid arthritis (RA) patient.

These collective findings, both in CD11c+/MHCII+/Ly6G+ neutrophil-DC hybrids emerging in GM- CSF-supplemented BM culture and CD66b+/HLA-DR+/CD86+ hybrids from synovial fluid of RA patients, suggested that hybrids retain several features of their neutrophil progenitors, and are distinguishable from conventional DCs in gene expression profile. Conversely, granulocyte- macrophage progenitor genes (ex. CD34), which are highly expressed in conventional mouse DCs and hybrid cells, were almost undetectable in the three populations from SF samples. Remarkably, the DC marker CD11c highly expressed in DCs and hybrid cells in GM-CSF-supplemented BM culture, showed a reversed trend of elevated expression in neutrophils derived from synovial fluid of RA patients. CD115, a macrophage marker dominantly expressed in DCs and hybrids, was consistent in expression between the two original types of sample (Figure 5B). Thus, given the comparison of the above genes in neutrophils, DCs, and neutrophil-DC hybrids in BM culture and clinic

synovial fluid sample, we may conclude that most sample sources presented a consistent profile of differentially-expressed genes in each cell type. Nevertheless, the existence of some gene expression diversity, especially in DC-dominant genes, implied surface phenotype heterogeneity of the three populations generated from various types of tissue. However, we can also reach the conclusion that the neutrophil-derived hybrid cells share several genes thought unique to either neutrophils or DCs, which may be related to the hybrids' role as simultaneously functional APCs and phagocytes.

Discussion

Several of our early studies demonstrated that neutrophils can differentiate into a previously- unrecognized leukocyte population exhibiting dual features of neutrophils and DCs, termed "neutrophil-DC hybrid" cells. This unusual population acquired the DCs markers (CD11c, MHCII, CD80, and CD86) while retaining some original neutrophil markers (Ly6G, CRCR2, and 7/4) as well [8]. They exhibited several features typical of DCs, such as dendritic morphology, probing motion, podosome formation, cytokine production, and presentation of foreign protein antigens to naïve CD4 T cells. In addition, these cells took on the role of professional phagocytes by means of extrusion of neutrophil extracellular traps (NETs), microbicidal actions, and recruitment of monocytes. Our findings implied that full maturation into PMNs with a short life or participation in host innate immunity is not the only option for all neutrophils. By testing a total of 61 cytokines, we found that the additional pathway of neutrophil destination was regulated by selected local cytokines, such as GM-CSF, IL-4, and INF-γ [19]. Correlated with our findings in mice, human neutrophils can also acquire MHC II expression when cultured in the presence of GM-CSF and/or INF-γ, as well as in some pathological conditions. MHC II expression was observed in a significant percentage (22-38%) of circulating blood neutrophils in patients after 2 weeks of INF-γ treatment [20] or 47-63% after 3 days in the presence of GM-CSF [21]. More importantly, neutrophil-DC hybrids were detected, albeit in varying and limited numbers, in many patients suffering different types of inflammatory disease [11,12]; similar cells have been observed within tumor micro-environments [14]. One might predict that the presence of GM-CSF and INF-γ in inflammatory lesion correlates very closely with the existence of such specialized neutrophil subsets.

As it is a disease comprising a large percentage of autoimmune conditions, rheumatoid arthritis can cause chronic inflammation of the joints and other areas of the body, with the possibility of permanent joint destruction and deformity. In response to chemoattractants released as part of the body's response to a falsely-perceived threat, namely autoantigens, infiltrating neutrophils appear in synovial fluid in abundance. In the RA model, neutrophil-depleting mAb treatment can protect mice against the inflammatory effects of K/BxN serum transfer, even reversing the inflammatory reaction in the joints [22]. These observations give rise to a critical role for neutrophils within the molecular pathology of RA in initiating and maintaining inflammatory processes in the joints. Once recruited into a given joint, the neutrophils become part of a local environment rich in molecules of the inflammatory milieu, such as IL-1β, TNF-α, GM-CSF, and IL-6 [23]. These stimuli are capable of not only modulation of neutrophil functions, such as priming, but also of propagating longer-lasting effects, namely the activation and perpetuation of certain genes expressed on neutrophils [15]. Several studies have failed to identify significant phenotypic differences in neutrophils from the blood of RA patients versus those of healthy controls. Fortunately, dramatic changes in protein expression levels are detectable in neutrophils isolated from the synovial fluid of RA patients, including APC-associated MHC Class II [16] and low levels of the costimulatory molecules CD80 and CD86. These findings highlight a novel role of the heretofore unknown neutrophil transdifferentiation taking place in diseased joints, which may be important or even essential to the initiation and propagation of RA pathology.

In the present study, surface expression of markers characteristic of neutrophils (CD66b) and DCs (HLA-DR, CD11c, CD40, CD80, CD83, and CD86) on SF neutrophils obtained from RA patients were measured by flow cytometry. We found a large population of cells with highly- expressed CD66b in all tested samples, which corroborated the previous findings that the neutrophil is the most abundant of all cells in the joints of patients with active rheumatoid arthritis [24]. Next, we gated the positive and negative cells based on their CD66 expression level and investigated several selected DC markers in synovial fluid that were also found in neutrophil-DC hybrids in GM-CSF culture [8] and within experimental inflammatory sites in mice [9]. In CD66-negative populations, a significant sub-population highly expressing HLA and either CD40 or CD86 emerged on the dot-plots. These observations suggested that DCs in synovial fluid from RA patients express characteristically high levels of HLA, CD40, and CD86 labeling. However, CD11c, CD80, and CD83 were relatively lower in expression. Ultimately, we detected a unique population with high level of HLA-DR, and CD40 or CD86 in CD66-positive neutrophils, which we previously defined as neutrophil-DC hybrid cells. The low expression of CD83 by hybrid cells in RA correlated the previous speculations that upregulation of MHC II and CD83 are not necessarily linked to each other, and CD83 expression can preferentially serve as a marker that transdifferentiation is occurring within the scope of acute inflammation [25]. Regardless of whether further experiments will corroborate this hypothesis, the phenotype results showed an expression profile of specific DC markers in the SF of RA patients, and made it feasible to isolate the hybrid population by FACS. When we used the staining combination, detailed as CD66b+/HLA-DR+/CD86+ or CD66b+/HLA-DR+/CD40+, the hybrid cells were detectable in all clinical samples, albeit at relatively low frequencies (from 0.1 to 1.5%). The variation between samples may be a result of the complexity of RA with regards to severity, time of onset, treatment, and symptoms, all of which occur in unique combinations in each RA patient.

Next, surface marker expression was confirmed at the transcriptional level by microarray data in three populations after sorting. In neutrophil-DC hybrids, mRNA for MHC class II (HLA-DRA, HLADRB1, HLA-DRB3, HLA-DRB4, and HLA-DRB5) and co-stimulatory molecules (CD40, CD80, and CD86) was comparable with that of DCs, although the CD66 signal was lower than that of neutrophils but higher than the DC population. Interestingly, within the CD66-positive neutrophil population, high levels of costimulatory factor CD86 mRNA were observed in some clinical samples (3/5), in which the MHC II was also differentially expressed at a low level. These findings showed that CD86 and MHC II expression can occur independently of each other in certain cases. Taken together, the results of our experiments demonstrated that some neutrophils in the joints of patients with RA begin to highly express DC markers (HLA, CD40, and CD86) at the protein synthesis and mRNA levels; this can then be detected by various methods, and the products may be purified.

Considering that neutrophils of the preferred mouse model differ in important aspects (such as antimicrobial repertoires and numbers in circulation) from their human counterparts, it is also important to compare current micro-array results in RA samples with our previous experimental data based on gene expression of mouse BM-derived neutrophil-DC hybrids, traditional DCs, and neutrophils. In those studies, the RNA was extracted for micro-array from neutrophil-DC hybrids. Ly6G- traditional DCs were purified from BM cultures [8] and neutrophils were freshly purified from BM [18]. Consistent with our published array data, most neutrophil-DC hybrids in synovial fluid from RA patient produced higher levels of CXCR2, CAMP, and G-CSFR mRNA than DCs did [8]. CXCR2, CXC receptor2, acting as a G protein-coupled receptor (GPCR) for interleukin 8 (IL-8), plays an essential role in acute inflammatory responses by mediating neutrophil recruitment [26] and NADPH oxidase-independent NET formation [27]. It also appears to be a neutrophil-specific marker, detectable almost exclusively within the Ly6G+ fraction of CD45+ hematopoietic cells in the peripheral blood and spleens of experimental mice [8]. CAMP, human cathelicidin-related antimicrobial peptide 18, when proteolytically processed to its active form LL-37, is secreted mainly by neutrophils and mediates bacterial killing [28] and apoptosis [29] in PMNs. Although the hybrids seemed more closely related to DC with regards to gene-expression profiles and morphological properties, the fact that the cells expressed neutrophil-dominant genes demonstrated that hybrids retain several unique features of neutrophils, which make them distinguishable from conventional DCs.

We may speculate that those unusual neutrophils, showing APC-like properties, play a protective role against infection through clearance of microbial invasion and presentation of various forms of foreign protein antigens to naïve CD4 T-cells during inflammation. They may also be involved in autoimmune diseases, digesting and attacking healthy tissue through the mechanism of CD4+ T-cell activation. Nevertheless, the exact function of hybrid emergence in inflammatory lesion remains to be elucidated due to the limitations of current experimental approaches, which cannot distinguish separation between the relative contributions of conventional neutrophils and neutrophil-DC hybrid cells. For example, systematic injection of anti-Ly6G mAb, which does not recognize monocytes or T cells, to deplete neutrophils has been widely used as a specific experimental method in mice as a way to investigate the role or roles of these cells in host defense, compared to administration of anti-Gr-1 mAb; the latter method depletes neutrophils, monocytes, and some CD8+ T cells, rendering it unfeasible for our experiments [30,31]. However, the expression of Ly6G can be detected on the surfaces of both mouse neutrophils and mouse hybrid cells. Thus, further studies are required to define the unique surface molecules(s) selectively expressed by neutrophil-DC hybrids for more selective depletion, molecules that by necessity must not be expressed by conventional neutrophil and DCs. Such works are in progress in our laboratory to identify the unique gene markers for hybrids in RA, sepsis, and renal- transplant patients, with our ultimate aim being new diagnostic and therapeutic approaches for immune dysfunctions that target this specific sub-population of hybrid cells.

Conclusion

To summarize the results of this paper, neutrophil-DC hybrid cells were identified and isolated from synovial fluid of RA patients, although it must be noted that the number of samples was limited. By means of micro-array analysis, the gene expression profile of hybrids was compared with those of neutrophils and DCs, and with the data obtained from experimental inflammatory mouse models. Our finding validated previous observations that transdifferentiation occurs in the joints of RA patients, suggesting an extended set of possible contributions for neutrophils and their descendant cells in the pathogenesis of RA and other inflammatory diseases. This also opens up a number of potential new treatments for an inflammatory and autoimmune disease that work on the cellular and molecular level, contingent on further experiments in this vein.

Disclosure

The authors have no conflicts of interest to disclose.

Acknowledgments

The authors thank Dr. Nezam Altorok and Dr. M. Bashar Kahaleh for their assistance in synovial fluid sample collection from patients with RA. This work was conducted in collaboration with and financially supported by Kyowa Hakko Kirin Co. Ltd.

References

1. Yang D, De la Rosa G, Tewari P, Oppenheim JJ (2009) Alarmins link neutrophils and dendritic cells. Trends Immunol 30: 531-537.
2. Serhan CN (2007) Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways. Annu Rev Immunol 25: 101-137.
3. Schmielau J, Finn OJ (2001) Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of T-cell function in advanced cancer patients. Cancer Res 61: 4756-4760.
4. Pelletier M, Maggi L, Micheletti A, Lazzeri E, Tamassia N, et al. (2010) Evidence for a cross-talk between human neutrophils and Th17 cells. Blood 115: 335-343.
5. Puga I, Cols M, Barra CM, He B, Cassis L, et al. (2011) B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. Nat Immunol 13: 170-180.
6. Fanger NA, Liu C, Guyre PM, Wardwell K, O'Neil J, et al. Activation of human T cells by major histocompatability complex class II expressing neutrophils: proliferation in the presence of superantigen, but not tetanus toxoid. Blood 89: 4128-4135.
7. Takashima A, Yao Y (2015) Neutrophil plasticity: acquisition of phenotype and functionality of antigen-presenting cell. J Leukoc Biol 98: 89-96.
8. Matsushima H, Geng S, Lu R, Okamoto T, Yao Y, et al. (2013) Neutrophil differentiation into a unique hybrid population exhibiting dual phenotype and functionality of neutrophils and dendritic cells. Blood 121: 1677-1689.
9. Geng S, Matsushima H, Okamoto T, Yao Y, Lu R, et al. (2013) Emergence, origin, and function of neutrophil- dendritic cell hybrids in experimentally induced inflammatory lesions in mice. Blood 121: 1690-1700.
10. Iking-Konert C, Csekö C, Wagner C, Stegmaier S, Andrassy K, et al. (2001) Transdifferentiation of polymorphonuclear neutrophils: acquisition of CD83 and other functional characteristics of dendritic cells. J Mol Med (Berl) 79: 464-474.
11. Hänsch GM, Radsak M, Wagner C, Reis B, Koch A, et al. (1999) Expression of major histocompatibility class II antigens on polymorphonuclear neutrophils in patients with Wegener's granulomatosis. Kidney Int 55: 1811-1888.
12. Iking-Konert C, Vogt S, Radsak M, Wagner C, Hänsch GM, et al. (2001) Polymorphonuclear neutrophils in Wegener's granulomatosis acquire characteristics of antigen presenting cells. Kidney Int 60: 2247-2262.

13. Davey MS, Morgan MP, Liuzzi AR, Tyler CJ, Khan MWA, et al. (2014) Microbe-specific unconventional T cells induce human neutrophil differentiation into antigen cross-presenting cells. J Immunol 193: 3704-3716.
14. Singhal S, Bhojnagarwala PS, O'Brien S, Moon EK, Garfall AL, et al. (2010) Origin and role of a subset of tumor-associated neutrophils with antigen-presenting cell features in early-stage human lung cancer. Cancer Cell 30:120-135.
15. Cross A, Bakstad D, Allen JC, Thomas L, Moots RJ, et al. (2005) Neutrophil gene expression in rheumatoid arthritis. Pathophysiology 12: 191-202.
16. Cross A, Bucknall RC, Cassatela MA, Edwards SW, Moots RJ (2003) Synovial fluid neutrophils transcribe and express class II major histocompatibility complex molecules in rheumatoid arthritis. Arthritis Rheum 48: 2796-2806.
17. Iking-Konert C, Ostendorf B, Sander O, Jost M, Wagner C, et al. (2005) Transdifferentiation of polymorphonuclear neutrophils to dendritic-like cells at the site of inflammation in rheumatoid arthritis: evidence for activation by T cells. Ann Rheum Dis 64:1436-1442.
18. Yao Y, Matsushima H, Ohtola JA, Geng S, Lu R, et al. (2015) Neutrophil priming occurs in a sequential manner and can be visualized in living animals by monitoring IL-1beta promoter activation. J Immunol 194: 1211-1224.
19. Geng, S, Matsushima H, Okamoto T, Yao Y, Lu R, et al. (2013) Reciprocal regulation of development of neutrophil-dendritic cell hybrids in mice by IL-4 and interferon- gamma. PLoS One 8: e82929.
20. Reinisch W, Tillinger W, Lichtenberger C, Gangl A, Willheim M, et al. (1996) In vivo induction of HLA-DR on human neutrophils in patients treated with interferon- gamma. Blood 87: 3068.
21. Mudzinski SP, Christian TP, Guo TL, Cirenza E, Hazlett KR, et al. (1995) Expression of HLA-DR (major histocompatibility complex class II) on neutrophils from patients treated with granulocyte-macrophage colony-stimulating factor for mobilization of stem cells. Blood 86: 2452-2453.
22. Wipke BT, Allen PM (2001) Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis. J Immunol 167: 1601-1608.
23. Feldmann M, Brennan FM, Chantry D (1990) Cytokine production in the rheumatoid joint: implications for treatment. Ann Rheum Dis 49: 480-486.
24. Kitsis E, Weissmann G (1991) The role of the neutrophil in rheumatoid arthritis. Clin Orthop Relat Res 265: 63-72.
25. Iking-Konert C, Wagner C, Denefleh B, Hug F, Schneider M, et al. (2002) Up-regulation of the dendritic cell marker CD83 on polymorphonuclear neutrophils (PMN): divergent expression in acute bacterial infections and chronic inflammatory disease. Clin Exp Immunol 130: 501-508.
26. Goncalves AS, Appelberg R (2002) The involvement of the chemokine receptor CXCR2 in neutrophil recruitment in LPS-induced inflammation and in Mycobacterium avium infection. Scand J Immunol 55: 585-591.
27. Marcos V, Zhou Z, Önder Yildirim A, Bohla A, Hector A, et al. (2010) CXCR2 mediates NADPH oxidase- independent neutrophil extracellular trap formation in cystic fibrosis airway inflammation. Nat Med 16:1018-1023.
28. Jann NJ, Schmaler M, Kristian SA, Radek KA, Gallo RL, et al. (2009) Neutrophil antimicrobial defense against Staphylococcus aureus is mediated by phagolysosomal but not extracellular trap-associated cathelicidin. J Leukoc Biol 86: 1159-1169.
29. Zhang Z, Cherryholmes G, Shively JE (2008) Neutrophil secondary necrosis is induced by LL-37 derived from cathelicidin. J Leukoc Biol 84: 780-788.
30. Daley JM, Thomay AL, Connolly MD, Reichner JS, Albina JE (2008) Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. J Leukoc Biol 83: 64-70.
31. Carr KD, Sieve AN, Indramohan M, Break TJ, Lee S, et al. (2011) Specific depletion reveals a novel role for neutrophil-mediated protection in the liver during Listeria monocytogenes infection. Eur J Immunol 41: 2666-2676.