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Chronic Psoriatic Skin Inflammation Leads to Increased Monocyte Adhesion and Aggregation

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Psoriasis patients exhibit an increased risk of death by cardiovascular disease (CVD) and have elevated levels of circulating intermediate (CD14++CD16+) monocytes. This elevation could represent evidence of monocyte dysfunction in psoriasis patients at risk for CVD, as increases in circulating CD14++CD16+ monocytes are predictive of myocardial infarction and death. An elevation in the CD14++CD16+ cell population has been previously reported in patients with psoriatic disease, which has been confirmed in the cohort of our human psoriasis patients. CD16 expression was induced in CD14++CD16+ classical monocytes following plastic adhesion, which also elicited enhanced β2 but not β1 integrin surface expression, suggesting increased adhesive capacity. Indeed, we found that psoriasis patients have increased monocyte aggregation among circulating PBMCs, which is recapitulated in the KC-Tie2 murine model of psoriasis. Visualization of human monocyte aggregates using imaging cytometry revealed that classical (CD14++CD16−) monocytes are the predominant cell type participating in these aggregate pairs. Many of these pairs also included CD16+ monocytes, which could account for apparent elevations of intermediate monocytes. Additionally, intermediate monocytes and monocyte aggregates were the predominant cell type to adhere to TNF-α and IL-17A-stimulated dermal endothelium. Ingenuity Pathway Analysis demonstrated that monocyte aggregates have a distinct transcriptional profile from single monocytes and monocytes following plastic adhesion, suggesting that circulating monocyte responses to aggregation are not fully accounted for by homotypic adhesion, and that further factors influence their functionality.

Psoriasis is a chronic inflammatory disease of the skin affecting 2–3% of the population of the United States in which expression is modified by susceptibility genes and environmental triggers (1). The pathogenesis of psoriatic tissue hyperplasia is thought to be driven by an interplay of macrophages, dendritic cells (DCs), and pathogenic and resident memory T cells, with enhanced representation of the IL-23–Th17/Th22 and IL-12–IFN-γ/TNF pathways (2, 3). In addition to an enormous negative impact on quality of life, psoriasis patients exhibit numerous comorbidities, including destructive psoriatic arthritis, stigmatization, depression and anxiety, inflammatory bowel disease, lymphoma, obesity, metabolic syndrome–associated conditions, and, notably, increased risk of early death from cardiovascular disease (CVD) (4–9). A mechanism has not yet been elucidated linking psoriasis pathogenesis and onset of CVD, but recent genome-wide association studies found that psoriatic individuals have common genetic variants that predispose them to increased risk of dyslipidemia, hypertension, and coronary artery disease, revealing an association of cardiovascular and metabolic disease genes with psoriasis (10).

Efforts to identify circulating inflammatory transducers of CVD revealed that increases in circulating intermediate monocyte subpopulations are Associated with CVD (11), acute ischemic heart failure (12), myocardial infarction (13), peripheral artery disease (14), and acute coronary syndrome associated with HIV (15). Within human peripheral blood, three distinct monocyte populations have been identified and genotyped: classical monocytes (CD14++CD16−), intermediate monocytes (CD14++CD16+), and nonclassical monocytes (CD14+CD16+) (16–22). Psoriasis patients, who also exhibit an increased risk of death by CVD, have been reported to have elevated levels of circulating CD16+ cells, which contain the intermediate monocyte population (23, 24). Induction of CD16 on the intermediate monocyte population can occur as a result of platelet interaction (25), which also increases monocyte adhesion to vascular endothelium and subsequent transendothelial migration (26). Indeed, circulating monocyte/platelet aggregates are considered a robust marker of platelet activation and indicator of coronary artery disease (27), ST segment elevation myocardial infarction (13), and acute myocardial infarction (28), as reviewed in van Gils et al. (29).

In murine CVD models, a proinflammatory monocyte subset (CD11b+Ly6C−) infiltrates murine atherosclerotic plaques and promotes atherogenesis (30) and also plays a role in myocardial...
infarction (31). Interestingly, in the skin-specific KC-Tie2 murine model of psoriasis, elevated levels of circulating CD11b^+Ly6C^hi cells are observed and precede the spontaneous formation of aortic root lesions. Moreover, these mice also develop a prothrombotic clotting phenotype (32), consistent with the idea that skin-contained chronic inflammation may have the capacity to promote atherothrombosis.

In this study, we demonstrate that psoriasis patients have both a relative and absolute increase in circulating monocyte aggregates as well as an increase in intermediate monocytes, correlating with an increase in disease severity assessed by the psoriasis area severity index (PASI), compared with healthy controls. Interestingly, control intermediate monocytes demonstrate increased adhesiveness to human dermal microvascular endothelial cells (HMVEC-D) following endothelial cell stimulation with proinflammatory cytokines known to be increased in psoriasis skin (TNF-α and IL-17A). Circulating monocyte-monocyte aggregates are also present in the KC-Tie2 murine psoriasis form model. We also show that monocyte aggregation in humans is associated with a distinct transcriptional profile and can occur in the presence or absence of platelets. Taken together, this data suggests a novel role for monocyte adhesion and subsequent aggregation as a potential link between the pathogenesis of psoriasis and CVD.

**Materials and Methods**

**Human subjects**

All studies of human subjects were approved by the Institutional Review Board of University Hospitals Case Medical Center (Cleveland, OH). Peripheral blood samples and/or punch biopsies were obtained from volunteer healthy controls and psoriasis patients following informed consent. Psoriasis patients were not on any systemic psoriasis medications, and those patients using any topical therapeutics discontinued use for at least 2 wk prior to entering the study. For patient demographics, see Supplemental Table I.

**Cell culture**

PBMCs were isolated from 23 controls and 19 psoriasis patients using ficoll-paque centrifugation, washed, and RBCs were lysed using ACK (Invitrogen, Carlsbad, CA) and then immediately stained for surface markers. Experiments were performed using total PBMCs and electronically gated for monocyte subpopulations. For adhesion studies, PBMCs were plated on tissue culture–treated six-well dishes for 30 min, 1 h, 4 h, or overnight in RPMI 1640 media supplemented with FBS and penicillin/streptomycin in 5% CO2 at 37°C.

**Flow cytometry**

Flow cytometric data collection was performed using a BD FACSAnia instrument and analyzed using FlowJo software (Tree Star, Ashland, OR). Monocytes were initially gated using a forward scatter (FSC) area (FSC-A) versus side scatter (SSC-A) discrimination plot and then analyzed for specific CD14 versus CD16 staining (see Supplemental Fig. 2). Stained cells were analyzed for CD14 versus CD16 scatter plot of doublets, each subset was plotted again as area-versus-aspect ratio of each respective channel. This allowed us to separate out homogeneous versus heterogeneous cell pairs. For example, a doublet that contained two CD14^+ cells would have a larger area on the SSC-A channel than would a doublet that contained one CD14^+ cell and one CD14^− cell (such as a T cell).

**Gene expression array**

RNA was extracted from flow-sorted CD14^+CD16^- singlet cells and CD14^+ doublet cells using the Qiagen RNasy Mini kit in combination with the Qiagen QIAshredder kit per the manufacturer’s protocol from psoriasis patients (n = 5) and controls (n = 5). A portion of the CD14^+CD16^- singlets were adhered for 4 h on tissue culture plastic before RNA extraction, cDNA was made using the RT^2 First Strand kit (SA Biosciences, Valencia, CA). cDNA was combined with RT^2 SYBR Green Master mix and dispensed into a PCR array for human extracellular matrix and adhesion molecules (Qiagen) with a 384 (4 × 96) E, G format. This data from this study have been deposited in National Center for Biotechnology Information’s Gene Expression Omnibus and are accessible through Gene Expression Omnibus series accession number GSE70327 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70327). Raw RT-PCR data were upsampled to SA Biosciences RT^2 Profiler PCR Array Data Analysis website (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php), where all samples were normalized to the housekeeping gene HPRT1. Each individual patient’s singlet monocytes served as the comparator for the test conditions of doublet monocytes and adhered monocytes. A heat map was generated using R: A Language and Environment for Statistical Computing (Vienna, Austria). For changes in the heat map, changes were calculated from ratios of doublet monocytes compared to singlet monocytes or adhered monocytes compared to singlet monocytes using the following Excel equation: =IF(value > 1, value/(1/value) – 1). Fold change values of >20 were changed to 20, and fold change values of <−20 were changed to −20 to normalize the range to most values and avoid an unclear gradient within the heat map. The psoriasis top network, representing the leukocyte extracellular matrix pathway, was generated through the use of Qiagen’s Ingenuity Pathway Analysis (IPA): Qiagen, Redwood City, CA: http://www.qiagen.com/ingenuity) with an average of all control CD14^- doublets as the comparator.

**Immunofluorescence**

Frozen 4-μm punch biopsies from involved psoriasis plaque of human volunteers were sectioned into 8-μm slices and four serial sections were mounted on each slide to provide proper controls for staining. Tissue sections were fixed in acetone for 10 min, washed in PBS buffer (HyClone/ThermoScientific, Waltham, MA), rehydrated using Ab diluent solution (MP Biomedicals, Santa Ana, CA), and blocked in 20% secondary isotype-specific serum (R&D Systems) for 30 min. Primary Abs were incubated either overnight at 4°C or for 1 h at room temperature dependent on the target and amplified using an isotype-specific corresponding secondary Ab for 1 h at room temperature (see Supplemental Table II). Nuclei were stained using one drop (60 μl) of Prolong Gold anti-fade reagent with DAPI (Molecular Probes/Life Technologies, Eugene, OR).

**Fluorescent image acquisition and analysis**

All images were acquired using the UltraVIEW VoX spinning disk confocal system (PerkinElmer, Waltham, MA), which is mounted on a Leica DMi6000B microscope (Leica Microsystems, Bannockburn, IL) at ×20 magnification.
Confocal images were collected using solid-state diode lasers, with 640, 488, 561, and 405 nm excitation light and with appropriate emission filters (see Supplemental Table II for Abs). All confocal images were analyzed using Volocity (PerkinElmer), MetaMorph Premier software (Molecular Devices, Sunnyvale, CA), and SigmaPlot (Systat Software, San Jose, CA). The white line designates the dermal–epidermal junction of skin sections.

Mouse studies

The KC-Tie2 binary, tet-repressible psoriasis mouse model, its genetic engineering, and the characterization of its skin and vascular phenotypes have been described at length previously (32, 34, 35). Mice spontaneously develop a chronic inflammatory skin phenotype following transgenic introduction of the angiopoietin receptor, Tie2, into keratinocytes (using the keratin 5 promoter). The skin inflammation phenocopies human psoriasis (35), is characterized by a robust Th1/Th17-skewed immune response, and is responsive to Abs targeting TNF-α or Ag cell depletion (34). Mice spontaneously develop systemic inflammation, elevated circulating CD11b+Ly6Cch monocytes, aortic root vascular inflammation, and are prothrombotic; these are reversed following targeted inhibition of the skin inflammation (32). Psoriasis-like inflammation was observed in all animals at the time of experiments. Spleens from male and female, adult, age-matched KC-Tie2 transgenic mice (1 y old mice on a CD1 outbred background, n = 10) and littermate controls (1 y old, n = 5) were removed and homogenized in serum-free media containing 50 μg/ml DNase I (Sigma-Aldrich, St. Louis, MO) and 2 mg/ml collagenase D (Roche, Basel, Switzerland). BCCI were lysed using ACK and then cells were pelleted, resuspended, and filtered twice through a 70-μm filter in wash buffer containing 5% FBS. The cells were immediately stained for the cell surface markers Ly6C–Alexa Fluor 700 (eBioscience, San Diego, CA) and CD11b–eFlour 450 (BD Biosciences), using 7-aminomatinomycin D to determine live cells. Cells were gated as previously published (32).

All animal protocols were approved by the Case Western Reserve University Institutional Animal Care and Use Committee and conformed to the American Association for Accreditation of Laboratory Animal Care guidelines.

Data analysis

Normality of distributions was tested using the Kolmogorov–Smirnov statistic, and upon nonrejection of this assumption, t tests for independent samples were used to compare mean values. Equality of variances was tested to select the appropriate resultant p values. The Mann–Whitney U test was used when the assumption of normality was not met. Correlations were estimated using Pearson correlation coefficients. Results are expressed as mean ± SE. Data analysis was done using SPSS v21, and graphs were generated using GraphPad Prism 6.

Results

Psoriasis patients have a higher percentage of circulating CD14++CD16 intermediate monocytes that correlates with disease severity

PBMC preparations were obtained from either psoriasis patients or healthy controls and analyzed for the percentage of circulating monocyte subsets (classical [CD14++CD16–], intermediate [CD14++CD16+], or nonclassical [CD14–CD16++]). Higher percentages of circulating intermediate monocytes were observed among psoriasis patients compared with controls (16.5 ± 2.7 versus 11.9 ± 1.4%, n = 19 and n = 23, respectively; p = 0.056) (Fig. 1A). Classical and nonclassical subsets showed no significant differences between psoriasis patients and healthy controls (65.5 ± 3.2 versus 70.2 ± 1.7%, CD14++CD16–, and 4.1 ± 0.59 versus 3.8 ± 0.42%, CD14–CD16++; Fig. 1B). Representative individual scatter plots are shown in Fig. 1C. Interestingly, the percentages of circulating classical and intermediate cells correlate with psoriasis disease severity (measured by the PASI) as shown in Fig. 1D. Whereas the classical subset negatively correlates with PASI (r = −0.541, p = 0.017), the intermediate subset positively correlates with PASI (r = 0.638, p = 0.003), and nonclassical monocytes do not demonstrate a significant correlation (r = −0.082, p = 0.738, data not shown). When absolute numbers of cells are calculated, psoriasis patients have increased numbers of total monocytes and doublets compared with healthy control individuals (Table I).

Monocyte doublets are increased in psoriasis patients compared with controls

We noted that a prominent population of cells was expressed within the intermediate CD14++CD16– gate in the scatter plot of psoriatic PBMCs (Fig. 2A). Back-gating this population revealed that these cells were larger on FSC-A versus SSC-A scatter plots and mapped to a region expected to contain doublets (Fig. 2B). Indeed, cell width–versus-height analysis on FSC of total events confirmed the accumulation of doublets (Fig. 2C, top row). Doublet discrimination analysis demonstrated that psoriasis patients have a >2.5-fold increase in total PBMC doublets compared with controls (Fig. 2D; 2.56 ± 0.54 versus 1.06 ± 0.17%, n = 19 and n = 23, respectively; p = 0.006). The top quartile of psoriasis patients (5 of 19) with increased doublets have a positive correlation that approaches significance with disease duration (r = 0.804, p = 0.101), suggesting that increased doublet percentage may be an additional indicator of disease severity. To ensure that doublet formation was relevant to in vivo psoriasis circulation, we also measured monocyte subset and doublet formation using whole blood, a technique commonly used to quantify the percentage of circulating monocyte subsets (36). As shown in Fig. 2C (bottom row), the increase in doublet formation can also be captured in whole-blood assays.

To confirm which cells form aggregates, we used an Amnis ImageStream flow cytometer to acquire an image of each cellular event. This analysis is capable of visualizing cellular events by capturing a photographic image that corresponds to each flow cytometric event acquired. PBMCs were stained using mouse anti-human CD14-allophycocyanin, CD16-FITC, and DAPI to discriminate among classical, intermediate, and nonclassical monocytes as shown in Fig. 3A. ImageStream analysis recapitulated our standard flow cytometric analysis confirming that monocyte doublets were 2-fold more likely in psoriatic PBMCs compared with healthy control samples (Fig. 3B). Interestingly, analysis of the doublets revealed that monocytes were capable of forming pairs with different subsets of lymphocytes. This included different monocyte subsets such as an intermediate/classical pair (a CD14++CD16– cell binding to a CD14++CD16– cell) (Fig. 3C, representative images in Fig. 3D) as well as monocytes binding to other lymphocytes including T cells (Fig. 3C, inset), although monocyte/NK cell binding was not observed. Although some monocyte doublet pairs contain platelets, doublets (monocyte/monocyte) can also be formed in the absence of platelets. In the intermediate gate, most doublets are represented by classical/nonclassical (CD14++CD16–/CD14–CD16++) pairs. Intermediate (CD14++CD16–) monocytes binding to other lymphocytes are also evident, although they represent a minority of the cells comprising the intermediate doublet population. Interestingly, both classical and intermediate monocytes exhibit the capacity to bind either CD14+ or CD16+ monocytes, in addition to other lymphocytes, although the classical monocytes appear to have an enhanced capacity over the intermediate cells to form these pairs (Fig. 3C).

Intermediate CD14++CD16– cells are detectable in psoriasis tissue

Although intermediate CD14++CD16– monocytes have been proposed to be DC precursors, the evolution/differentiation of these cells remains controversial (37). As shown in Fig. 4, intermediate monocytes (yellow arrows) can indeed be detected in psoriasis-involved papillary dermal perivascularure, as confirmed by colocalization with CD31 (Supplemental Fig. 1A), indicating that they do gain entry into lesional tissue. Interpersonal variation in the number of intermediate monocytes was observed; however, all patients had detectable intermediate monocytes within lesional skin. Some monocytes appear to be undergoing differentiation to

monocytes to...
DCs as evidenced by coexpression with DEC-205 (Supplemental Fig. 1C), or to macrophages based on CD68 coexpression (Supplemental Fig. 1B). Given the known criticality of T cells in psoriasis, we also identified CD3+ cells present in the plaque adjacent to, but not overlapping with, CD14+ cells (Supplemental Fig. 1D).

**FIGURE 1.** CD14+CD16+ (intermediate) cells are increased in psoriasis patients compared with healthy controls. (A) The percentage of intermediate monocytes (CD14+CD16+) is increased in psoriasis patients (n = 19, □) when compared with healthy controls (n = 23, ■; p = 0.056) whereas (B) the percentage of classical (○, ●) and nonclassical monocytes (△, ▲) do not differ between psoriasis patients and healthy controls. (C) Representative flow plots showing gates and population distribution. (D) Classical monocytes (red, ○) negatively correlate with disease severity measured by PASI (r = −0.541, p = 0.017), whereas intermediate monocytes (blue, □) positively correlate with disease severity (r = 0.638, p = 0.003).
Monocyte modulation of CD16 following adherence

Based on our results demonstrating increased intermediate monocytes in psoriasis circulating blood and tissue and the participation of CD14+ cells in monocyte/monocyte and monocyte/lymphocyte doublets, we anticipated that these monocytes would demonstrate increased adhesiveness. Indeed, exposure of monocytes to tissue culture plastic significantly increases the expression of CD16, confirming previously published observations (23). The classical monocyte population is significantly diminished, and most monocytes take on an intermediate monocyte phenotype beginning as early as 4 h and becoming nearly exclusively CD14++CD16+ following overnight adherence to plastic (Fig. 5A). Interestingly, monocytes derived from either psoriasis or healthy control peripheral blood exhibited the capacity to upregulate CD16 expression upon binding. To examine CD16 induction on a more physiologically relevant substrate, we cocultured control monocytes on prestimulated HMVEC-D (TNF-α and IL-17A) and compared them to monocytes cultured on unstimulated HMVEC-D. After 1 h, the supernatants of unstimulated HMVEC-D (Fig. 5B, middle panels) or stimulated HMVEC-D (Fig. 5B, right panels) were isolated and stained for CD14 and CD16 expression on monocytes. Although a brief exposure to endothelial cells did not induce CD16 to the same extent as did culturing on plastic, intermediate monocytes exhibited an average 2-fold increase in adhesion to stimulated HMVEC-D, based on monocyte number in the supernatant population, compared with unstimulated HMVEC-D (23.9 ± 4.5 versus 12.4 ± 1.2%, respectively, n = 4, p = 0.029; representative images in Fig. 5B, middle and right panels, supernatant intermediate population).

Psoriatic classical monocytes have increased β2 integrin expression at baseline

After determining that monocytes are capable of forming homogeneous pairs, and adhesion to plastic significantly increased CD16 expression, we asked whether psoriasis classical monocytes (Fig. 6, solid red lines; n = 3) had a different adhesion marker profile compared with classical cells from healthy controls (Fig. 6, solid blue lines; n = 3) at baseline. Psoriasis patients had moderately increased surface expression of the β2 integrins CD11b (Fig. 6A) and CD11c (Fig. 6B) with a slight elevation of CD18 (Fig. 6C, Table I. Absolute cell count

<table>
<thead>
<tr>
<th></th>
<th>Doubles/PMBC</th>
<th>Monocytes/Singlet PMBC</th>
<th>Classical Monocytes</th>
<th>Intermediate Monocytes</th>
<th>Nonclassical Monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.83 ± 0.12</td>
<td>18.8 ± 1.93</td>
<td>12.9 ± 1.38</td>
<td>2.46 ± 0.40</td>
<td>0.69 ± 0.10</td>
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<tr>
<td>Psoriasis</td>
<td>2.53 ± 0.82</td>
<td>32.3 ± 15.9</td>
<td>21.6 ± 11.2</td>
<td>4.89 ± 2.06</td>
<td>1.11 ± 0.45</td>
</tr>
</tbody>
</table>

Absolute cell counts were determined by percentage of monocytes/total PBMCs.

1 Defined based on an FSC-W versus FSC-H analysis of all events.
2 Monocytes defined on an FSC-A versus SSC-A analysis from the singlet events.
3 Defined as CD14++CD16+ from the monocyte and singlet gates.
4 Defined as CD14++CD16+ from the monocyte and singlet gates.
5 Defined as CD14+CD16++ from the monocyte and singlet gates.

Psoriatic patients have increased total doublets within PBMCs. (A) The prominent intermediate monocyte population in psoriasis patients was selected and in (B), cells were overlaid electronically into a monocyte scatter gate as shown in the FSC-A versus SSC-A plot. (C) Most cells were doublets, as shown in representative plots of FSC-W versus FSC-H, demonstrating singlet and doublet populations in PBMCs (n = 42) and whole blood (n = 8). (D) Analysis of all patients demonstrated that the total doublet percentage is significantly increased in psoriasis patients when compared with controls (○ versus ●, n = 19 and n = 23; respectively; p = 0.006).
Table II), whereas psoriasis and control patients had similar levels of ICAM-1 (Fig. 6D), VCAM-1 (Fig. 6E), and VLA-4 (Fig. 6F) expression. Isotype control curves are shown in gray.

Although control and psoriasis classical monocyte subsets up-regulate CD11b and CD11c at baseline, control monocytes induce greater upregulation of these markers after a 30-min adhesion

FIGURE 3. Amnis ImageStream visualization of cell surface expression of CD14-allophycocyanin and CD16-FITC and monocyte/monocyte doublets. (A) Representative images of each type of monocyte (classical, intermediate, and nonclassical) as imaged by the Amnis ImageStream cytometer (original magnification ×60). CD14-allophycocyanin (red) is expressed on the surface of the classical cells, CD16-FITC (green) is expressed on the surface of the nonclassical cells, whereas both CD14 and CD16 (orange) colocalize to the surface of the intermediate monocyte. (B) Psoriasis patients (n = 3) have increased monocyte doublets when compared with controls (n = 3) (filled bar versus open bar, respectively; as expressed by a ratio of % monocyte doublets/ % monocyte singlets). (C) Monocytes form aggregate pairs in the form of monocyte/monocyte or monocyte/lymphocyte. The classical CD14⁺ cells participate in the most doublet formation as a homogeneous pair (CD14⁺ binding to another CD14⁺) or as a heterogeneous pair (CD14⁺ binding to a lymphocyte). The CD14⁺ cells within the intermediate doublet gate also participate in homo- and heterogeneous pairs, but not to the same extent. Additionally, double-positive CD14⁺CD16⁺ monocytes participate in binding to CD3⁺ lymphocytes [as shown in (D)]. Nonclassical CD16⁺ cells rarely participate in homogeneous doublet pairs. (D) Representative images of the doublet pairs described in (C). Platelets (labeled with CD42b-PE [pink]) participate in doublet formation but are not necessary for monocyte/monocyte aggregation.
psoriasis patients (blue), acquired at an original magnification phycocyanin (red) and CD16-FITC (green) with nuclei stained by DAPI plaques. Frozen involved human psoriatic plaque stained for CD14-allo-CD14++CD16+ (intermediate) monocytes. Representative image from a pso-matrix and adhesion array) in both healthy controls (array specific for adhesion molecules (SABiosciences extracellular and monocytes forming doublets in peripheral blood using an RNA glet classical monocytes adhered to tissue culture plastic for 4 h), we compared the mRNA expression profiles of singlet, doublet, and adhered monocytes profile for singlet, doublet, and adhered monocytes PCR analysis demonstrates a different mRNA expression profiles were used as the comparator. A comparison of the average psoriasis classical singlet monocytes to the average expression of control classical singlet monocytes also revealed a distinct gene expression profile (Fig. 7B), although the changes were not as robust as those observed between doublet and adherent cell populations. Analysis of the psoriasis versus control doublets indicates up-regulation of several integrins, i-selectin (SELL), extracellular matrix proteins (VCAN, FN1, HAS1), and TGFβ1 (Fig. 8A). IPA analysis of the psoriasis doublet population identified the leukocyte extravasation pathway (shown graphically in Fig. 8B) as the top canonical pathway. Network analysis from this pathway identified several directly upregulated α-integrins (ITGAV, ITGAL, ITGAM, ITGA4, ITGA5), β-integrins (ITGB1, ITGB2, ITGB3, ITGB5), and cellular adhesion molecules (ICAM1, VCAM1) as well as several imputed genes of interest in psoriasis versus control doublets, including IL-1, the NF-κB complex, the IL-12 complex, and genes known to play a role in cell surface adhesion such as fibrinogen, fibrin, collagen type II, and focal adhesion kinase.

**Monocyte doublets are increased in KC-Tie2 mice**

To address what mediates the doublet formation, we used the KC-Tie2 skin-specific psoriasisform mouse model. KC-Tie2 mice have been previously shown to develop elevated systemic monocytes and neutrophils compared with wild-type (WT) controls (0.79 ± 0.17 versus 0.20 ± 0.08%, n = 10 and n = 5, respectively; p = 0.008; Fig. 9B, representative images in Fig. 9A), indicating that chronic skin-specific inflammation may influence circulating monocyte aggregation.

**Discussion**

Psoriasis is an immune-mediated inflammatory autoimmune disease (IMIAD) that has been demonstrated at the epidemiologic level to place patients at a higher risk for cardiovascular complications (6). The linkage of a number of IMIADs to CVD points to a common pathology, but there is a major gap in understanding how cellular inflammation at distant sites predisposes vascular
tissue to CVD. The advanced state of validation of specific psoriasis pathogenesis pathways via biologic therapies provides a unique opportunity to link pathomechanisms of IMIADs with CVD. Intervention with current biological therapeutics for psoriasis may be dependent on LFA-1-expressing leukocytes (myeloid cells and T cells), TNF-producing...
cells (monocytes, T cells, others), IL-23 (monocytes and DCs), and IL-17 (Th17 cells) (38–44). In humans, a newly defined intermediate monocyte subset (CD14++CD16+) is predictive of CVD, myocardial infarction, and death (11–13). Several publications have implicated CD14++CD16+ intermediate cells as critical mediators of inflammation (45–47). Importantly, there is compelling epidemiologic evidence connecting increases in intermediate monocytes as predictive of fatal cardiovascular complications (11) as well as compelling epidemiologic evidence connecting psoriasis to cardiovascular risk (4, 5). Several relevant review articles have described the critical relationship among monocytes and CVD (30, 48), and the concept of chronic inflammation driving cardiovascular outcomes has been validated in numerous murine models, including one psoriasiform mouse (32, 49, 50). The potential cellular mechanisms connecting psoriasis with elevated CVD risk has not been definitively addressed. As elevated levels of the intermediate subset have been shown to contribute to CVD (51), we hypothesized that increases in this CD14++CD16+ subset seen in human psoriasis patients may also mediate a link between psoriasis disease pathology and its associated CVD comorbidities. Increased monocyte/platelet aggregates in psoriasis patients suggest that circulating monocytes in these individuals may have increased adhesive properties and may play a potential role in the common pathology between psoriasis progression and CVD.

In this study, we asked whether psoriasis patients possessed elevated levels of intermediate monocytes and whether these monocytes demonstrated elevated adherence properties compared with healthy controls. Increased levels of intermediate (CD14++CD16+) cells in psoriasis circulation confirms a recent study among psoriasis patients (24) as well as a previously reported increase in total CD16++ cells in patients with psoriatic arthritis (23). We demonstrate in the present study that intermediate monocytes correlate with disease severity, are present in involved psoriatic plaque tissue, and preferentially bind to stimulated endothelial HMVEC-D. Upon back-gating of the circulating intermediate monocyte population, we noted that many of these cells appear consistent with larger cell populations judged by SSC. Additionally, when areas where doublets appear were included in the gating strategy, the intermediate

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**FIGURE 6.** Increased $\beta_2$ integrin expression on psoriasis classical monocytes. At baseline, psoriasis patients (solid red lines; $n = 3$) demonstrate increased surface expression of (A) CD11b and (B) CD11c with a slight upregulation of (C) CD18 on the classical population when compared with controls (solid blue lines; $n = 3$). After a 30 min plastic adhesion, control (dashed blue lines) and psoriasis (dashed red lines) classical monocytes upregulate (A) CD11b, (B) CD11c, and (C) CD18, whereas there is no change in (D) ICAM-1, (E) VCAM-1, or (F) VLA-4. Isotypes are shown on all panels in gray.
population was more prominent in these larger FSC areas. Because intermediate monocytes are thought to contribute to, or correlate with, numerous comorbid conditions for psoriasis, we hypothesized that doublets made up of two intermediate monocytes would be predominant in psoriasis pathology and plaque formation.

Using Amnis ImageStream technology, we demonstrated that monocytes can form monocyte/monocyte and monocyte/lymphocyte pairs in the presence or absence of platelets. Specifically, the predominant monocyte/monocyte doublet pairs consist of one CD14++CD16- classical monocyte binding to either another CD14++CD16- classical monocyte, a CD14++CD16+ intermediate monocyte, or a lymphocyte. To better understand which adhesion molecules may mediate the monocyte doublet formation, we screened control and psoriasis PBMCs using a panel of typical surface adhesion markers. Monocytes from psoriasis patients express moderately increased levels of $\beta_2$ integrins at baseline levels and do not upregulate expression of these adhesion markers to the same extent as healthy control monocytes upon adherence to plastic, suggesting that psoriatic monocytes may be previously primed by the circulating psoriasis milieu and are not able to be further stimulated. This potential priming and baseline elevation of CD11b and CD11c may lead to the observed increase in circulating monocyte/monocyte doublets of psoriasis patients. Additionally, coculture of control monocytes on TNF-α- and IL-17A–stimulated HMVEC-D results in an average 2-fold increase in adhesion of intermediate monocytes to stimulated HMVEC-D when compared with monocytes cultured on unstimulated HMVEC-D, indicating that myeloid cell interaction with activated psoriatic endothelium may contribute to observed enhanced adhesiveness.

### Table II. Mean fluorescence intensity changes

<table>
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<th>Adhesion Molecule</th>
<th>Baseline: Psoriasis/Control</th>
<th>Control: 30 min/Baseline</th>
<th>Psoriasis: 30 min/Baseline</th>
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<td>ICAM-1</td>
<td>1.0</td>
<td>1.3</td>
<td>1.2</td>
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<tr>
<td>CD11b</td>
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<td>1.1</td>
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*Change in mean fluorescence intensity of psoriasis classical monocytes compared with control classical monocytes. These cells were not adhered to plastic.

*Change in mean fluorescence intensity of control classical monocytes after a 30-min adhesion relative to baseline expression.

*Change in mean fluorescence intensity of psoriasis classical monocytes after a 30-min adhesion relative to baseline expression.

FIGURE 7. Gene networks in doublets and adhered classical monocytes. (A) Heat map of different gene expression patterns of CD14+ doublets (Dblt) and tissue culture–adhered classical singlet monocytes (Adh) in control (C; $n=5$) and psoriasis (P; $n=5$) individuals. Each individual patient’s singlet classical monocytes were used as the comparator. (B) Heat map of gene expression patterns of psoriasis singlet classical monocytes. An average of the classical singlet monocytes was used as the comparator.
To further define the gene expression pattern of classical singlet monocytes, doublets containing CD14+ cells, and monocytes postadherence, we used an mRNA expression array specific for extracellular matrix and adhesion gene expression and compared monocytes from doublet pairs or monocytes postadherence on tissue culture plastic to singlet classical monocytes. After adherence to tissue culture plastic, singlet classical monocytes isolated from healthy individuals upregulate genes involved in cellular adhesion.

**Figure 8.** Gene network of psoriasis doublets. (A) Psoriasis doublet gene network generated using the average of all psoriasis doublets (n = 5); an average of all control doublets (n = 5) is the comparator. (B) Leukocyte extravasation signaling pathway network of psoriasis doublets generated by IPA.

**Figure 9.** Monocyte doublets are increased in KC-Tie2 mice. (A) Representative figures of doublet gating strategy. Percentages represent doublets within the CD11bLy6Chi gate. (B) Splenic doublets are significantly increased in KC-Tie2 mice (n = 10) when compared with littermate WT controls (n = 5) (p = 0.008).
including integrins (ITGA3, ITGA7, ITGA8), a cadherin (CDH1), and VCAM-1 to a greater extent than do psoriasis monocytes, suggesting some disease-specific adhesion gene response profiles. Singlet plastic-adherent classical monocytes from both control and psoriasis patients upregulate several matrix metalloproteinases (MMP-1, -2, -10, and -14), thrombospondin, and secreted phosphoprotein 1 while downregulating P-selectin (SELP), indicating that plastic adhesion is distinct from cell–cell adhesion. The observed upregulation of MMPs in plastic adhesion when compared with the doublet cell populations suggests that the cell–cell interaction of monocyte/doublet pairs initiates signaling pathways that are distinct from a monocyte plastic-adherence phenotype. MMPs may play an important role in this adhesion as they are known to participate in atherosclerotic plaque stability (52) and are predominantly upregulated upon cellular contact with extracellular matrices. A major difference in gene expression patterns among psoriasis patients compared with healthy individuals occurs primarily following adhesion of classical monocytes to tissue culture plastic.

A readily apparent difference between monocytes in doublet pairs compared with monocytes after plastic adherence upregulation in doublets of integrins, saccoglycan, collagen type VI, α1, and α2, and disintegrins. Interestingly, upregulation of CD56 (NCAM1) is also observed in the doublet pairs compared with plastic-adherent monocytes. CD56 expression may indicate upregulation of the CD56+ monocyte population (53), previously demonstrated in other autoimmune disorders (54). Specific genes upregulated in doublet pairs, such as ITGA3 and ADAMTS1 (a disintegrin), suggest that integrins may participate in the cell–cell adhesion we observed using Amnis imaging technology. Although the function of ADAMTS1 in monocytes is unclear, one report showed that ADAMTS1 expression can be induced during monocyte to macrophage differentiation (55). Interestingly, a polymorphism in the ADAMTS1 allele has been linked to an increased risk of fatal coronary disease, indicating that it may play a role in mediating CVD (56).

IPA pathway network analysis of the leukocyte extravasation pathways in psoriasis doublets identified several imputed genes of interest, including genes known to play a role in cell surface adhesion such as fibrin, fibrinogen, a collagen, and focal adhesion kinase, while also including molecules known to participate in important signal transduction pathways impinging upon STAT3 regulation such as the NF-κB signaling complex. At baseline, comparison of singlet classical monocytes from psoriasis and healthy control patients demonstrates a distinct gene expression pathway in psoriasis monocytes that may account for their increased propensity to form doublets.

To determine what was mediating the doublet formation, we took advantage of the KC-Tre2 mouse, a psoriasiform model that exhibits chronic skin-specific inflammation and is known to have increases in circulating monocytes and thrombosis formation, as well as to develop aortic root vascular lesions. We show in this study that these mice also have increased circulating monocyte/monocyte doublets, suggesting that skin inflammation may drive cell differentiation and aggregation, although the precise mechanisms mediating this outcome require further study. Collectively, the data suggest that cells forming doublets in circulation upregulate distinct adhesion molecules that potentiate cellular adhesion and may increase the risk of cardiovascular comorbidities observed in psoriasis patients.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1: CD14⁺CD16⁺ co-localization with additional cell markers

(A) Frozen involved human psoriatic plaque stained for CD14 (red), CD16 (green), and CD31 (blue) to identify vasculature (n=3). (B) Frozen involved human psoriatic plaque stained for CD14 (red), CD16 (green), and the macrophage marker, CD68 (blue) (n=2). (C) Frozen involved human psoriatic plaque stained for CD14 (red), CD16 (green), and the DC marker, DEC-205 (blue) (n=2). (D) CD14⁺⁺ cells do not co-localize with CD3⁺ cells in involved psoriasis tissue. Frozen involved human psoriatic plaque stained for CD14 (red) and CD3 (green) with nuclei stained by DAPI (white) (n=3). In all sections, yellow arrows indicate double positive CD14/CD16 staining while white arrows indicate triple positive cells.
Supplemental Figure 2: Gating Strategy

Supplemental Figure 2: Monocyte and doublet gating strategy. (A) All PBMC events are gated on FSC-W vs. FSC-H to separate singlets from doublets. (B) Monocytes are electronically selected based on their SSC-A vs. FSC-A profile. (C) The left side of the classical population gate is determined based on CD14 isotype and the upper limit is determined by CD16 isotype. The intermediate population gate is also determined by CD14 isotype combined with all events above the CD16 isotype. The non-classical population is determined by the CD14 isotype for the right side of the gate and negative events for the left side of the gate. (D) Representative images obtained on the Amnis Imagestream cytometer of cells in the doublet gate post-sort demonstrating the doublet population used in subsequent transcriptomic analyses. Dotted line in panel A indicates doublet gate used in sort.
Supplemental Table I: Demographics

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*Note: One of the psoriasis patients from the PCR cohort (first on the heatmap) was on Soriatine at the time of the blood draw for a non-psoriasis condition*
Supplemental Table II: Antibodies used in Flow Cytometry and Immunofluorescence

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