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Chronic Psoriatic Skin Inflammation Leads to Increased Monocyte Adhesion and Aggregation

Jackelyn B. Golden,*‡ Sarah G. Groft,* Michael V. Squeri,* Sara M. Debanne,‡ Nicole L. Ward,*‡§ Thomas S. McCormick,*‡§ and Kevin D. Cooper*‡,§,¶

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 singlet 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. The Journal of Immunology, 2015, 195: 2006–2018.

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+Ly6G−) infiltrates murine atherosclerotic plaques and promotes atherogenesis (30) and also plays a role in myocardial...
inflammation (31). Interestingly, in the skin-specific KC-Tie2 murine model of psoriasis, elevated levels of circulating CD11b+Ly6C<sup>hi</sup> 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 psoriasisform 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 24 h 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, or 4 h or overnight in RPMI 1640 media supplemented with FBS and penicillin/streptomycin in 5% CO<sub>2</sub> 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) area (SSC-A) discrimination plot and then analyzed for specific CD14 versus CD16 staining (see Supplemental Fig. 2 for gating strategy and Supplemental Table II for Ab clone and source). Doublet analysis was performed by gating FSC width (FSC-W) versus SSC height (FSC-H) on total events. For experiments that required sorted monocytes, samples were sorted using an 85-psi pressure of 45 psi. Surface adhesion analysis of CD11b, CD11c, CD18, VLA-4, VCAM-1, and ICAM-1 was performed on gated classical monocyte populations. 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% CO<sub>2</sub> at 37°C.

Whole blood method

Whole blood (50 μl) from four healthy controls and four psoriasis patients was stained with mouse anti-human CD14 and CD16 for 15 min at room temperature. The blood was then lysed at room temperature with 450 μl 1 X FACS lysing solution (BD Biosciences, Franklin Lakes, NJ) for 12 min and analyzed immediately by flow cytometry.

Monocyte and HMVEC-D coculture experiments

Monocytes were negatively selected from PBMCs using the Pan Monocye kit (Miltenyi Biotec, Cologne, Germany). HMVEC-D (Lonza, Basel, Switzerland) were cultured in complete media (Lanza) at 37°C, 5% CO<sub>2</sub>, and IL-17A (100 ng/ml; R&D Systems) to mimic a psoriatic and cardiovascular cytokine profile, as published in Hot et al. (33). The cells were then rested for 30 min before addition of monocytes. HMVEC-D were used at P2–P3 and 90–100% confluence in a six-well dish. Four to 5 million monocytes were plated per well and adhered for 1 h before harvesting the adherent and supernatant fractions and staining for CD14 and CD10 surface markers.

Imaging flow cytometry

Heterogeneous doublet population data were collected using an Amnis ImageStream cyrometer and analyzed using IDEAS software v6.0. PBMCs were stained for surface expression of CD14, CD16, CD42b, CD3, and CD56 (for Abs, see Supplemental Table II). Events were first selected on focused events. Next, to gate singlets versus doublets, the area-versus-aspect ratio of the brightfield channel was plotted. Doublets were defined as having an aspect ratio &lt;0.6 with an area of 100–400 singlets and aspect ratio of 0.6 with an area of 100–400 singlets. Doublets were then separately gated into CD14-allophycocyanin versus CD16-FTC for three monocyte subset analyses. From the 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<sup>+</sup> cells would have a larger area on the CD16 channel than a doublet that contained one CD14<sup>+</sup> cell and one CD<sup>+</sup> cell (such as a T cell).

Gene expression array

RNA was extracted from flow-sorted CD14<sup>+</sup>CD16<sup>+</sup> singlet cells and CD14<sup>+</sup> doublet cells using the Qiagen RNeasy 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<sup>+</sup>CD16<sup>+</sup> singlets were adhered for 4 h in tissue culture plastic before RNA extraction. cDNA was made using the RT<sup>2</sup> First Strand kit (SA Biosciences, Valencia, CA). cDNA was combined with RT<sup>2</sup> SYBR Green Master mix and dispensed into a PCR array for human extracellular matrix and adhesion molecules (Qiagen) with a 384 (4 x 96) E, G format. The data discussed in 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?token=GSE70327). Raw RT-PCR data were up- or down-regulated according to SA Biosciences RT<sup>2</sup> 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 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 &gt; 1, value/(1/ value)- 1). Fold change values of &gt;20 were changed to 20, and fold change values of &lt;–20 were changed to –20 to normalize the range to most values and avoid an unclear gradient within the heat map. The psoriasis top down network, representing the leukocyte extracellular matrix pathway, was generated through the use of Ingenuity Pathway Analysis (IPA; Qiagen, Redwood City, CA; http://www.qiagen.com/ingenuity) with an average of all control CD14<sup>+</sup> doublets as the comparator.

Immunofluorescence

Frozen 4-mm 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/Thermo Scientific, Waltham, MA), rehydrated using Ab diluent solution (MP Biomedical, 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 of 4′,6-Diamidino-2-phenylindole (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 Velocity (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 psoriasisform 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+Ly6C<sup>hi</sup> 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). RBCs were lysed using 7-aminoactinomycin D to determine live cells. Cells were gated as [CD14++CD16<sup>+</sup>], or nonclassical [CD14+CD16++]. Higher percentages of circulating intermediate monocytes were observed (Fig. 1A). Classical and nonclassical subsets showed no significant differences (data not shown). When absolute numbers of cells are calculated, psoriasis patients have a higher percentage of circulating CD14<sup>+</sup>CD16<sup>+</sup> intermediate monocytes that correlates with disease severity (measured by the PASI) as shown in Fig. 1D. Scatter plots are shown in Fig. 1C. Interestingly, the percentages of CD14<sup>+</sup>CD16<sup>+</sup> monocytes binding to other lymphocytes are also increased in psoriasis patients compared with controls (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 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 collecting 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 psoriasis 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<sup>+</sup>CD16<sup>+</sup> cell binding to a CD14<sup>+</sup>CD16<sup>+</sup> 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<sup>+</sup>CD16<sup>-</sup>/CD14<sup>+</sup>CD16<sup>+</sup>) pairs. Intermediate (CD14<sup>+</sup>CD16<sup>-</sup>) 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<sup>+</sup> or CD16<sup>+</sup> 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<sup>+</sup>CD16<sup>-</sup> cells are detectable in psoriasis tissue

Although intermediate CD14<sup>+</sup>CD16<sup>-</sup> 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 perivascular, 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
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).
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 cultivating 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>Doublets/PBMC</th>
<th>Monocytes/Singlet PBMC</th>
<th>Classical Monocytes</th>
<th>Intermediate Monocytes</th>
<th>Nonclassical Monocytes</th>
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<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>
</tr>
<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>
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Absolute cell counts were determined by percentage of monocytes/total PBMCs.

Doublets defined based on an FSC-W versus FSC-H analysis of all events.

Monocytes defined on an FSC-A versus SSC-A analysis from the singlet events.

Defined as CD14+CD16+ from the monocyte and singlet gates.

Defined as CD14+CD16+ from the monocyte and singlet gates.

Defined as CD14+CD16++ from the monocyte and singlet gates.

2010 MONOCYTE ADHESION IN PSORIASIS

FIGURE 2. Psoriasis 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
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 plastic (Fig. 7A, heat map). As shown, doublet monocyte pairs (both psoriasis and healthy controls) express an upregulated cluster of genes distinct from postplastic adherent monocytes, including integrins (ITGA3, ITGA6, ITGB3), disintegrins (ADAMTS1, ADAMTS8, ADAMTS13), a matrix metalloproteinase (MMP11), collagens (COL5A1, COL6A1, COL6A2), and other cellular adhesion molecules (NCAM1, CDH1, LAMA2). Similarly, postplastic adherent monocytes have a unique upregulated gene set compared with baseline singlet monocytes and monocyte doublets that included numerous matrix metalloproteinases (MMP1, MMP2, MMP9, MMP10, MMP14), fewer integrins (ITGA7), a laminin (LAMB3), and an adhesive glycoprotein (THBS1). Interestingly, adhered singlet monocytes from healthy control individuals also appear to downregulate several cellular adhesion genes including an ICAM (ICAM-1), a sarcoglycan (SGCE), an extracellular matrix protein (KAL1), and a laminin (LAMA3) when compared with psoriasis adhered singlet monocytes. Each individual patient’s classical singlet monocytes 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 upregulation 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 (ITGA7, ITGAL, ITGAM, ITG4, ITG5), 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 psoriasiform mouse model. KC-Tie2 mice have been previously shown to develop elevated systemic monocytecytosis comprised of circulating, proinflammatory CD11b+Ly6C− monocytes (32); this precedes the spontaneous development of aortic root vascular inflammation. This CD11b+Ly6C− monocyte population has been previously correlated with the classical and intermediate human monocyte populations (22), and thus we were curious whether KC-Tie2 mice would also demonstrate increases in circulating monocyte/monocyte doublets. Similar to our observations in psoriasis patient blood, KC-Tie2 mice also demonstrated an ∼4-fold increase in monocyte/monocyte doublet formation compared with wild-type (WT) controls (0.79 ± 0.17 versus 0.20 ± 0.08%, n = 10 and n = 5, respectively; p = 0.01; 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...
FIGURE 5. Upregulation of CD16 expression and enhanced adhesion. (A) Representative images of PBMCs at baseline (left panel) were adhered for 4 h (middle panel) or overnight (right panel) to plastic and then stained for CD14 and CD16 (n = 3). After 4 h, expression of CD16 begins to increase; after overnight culture almost the entire classical population has upregulated CD16 and transitioned into the intermediate gate. (B) Representative images of monocyte gating of negatively selected control monocytes at baseline (left panels) and after 1 h culture with unstimulated endothelial HMVEC-D (middle panels) and HMVEC-D stimulated with TNF-α (1 ng/ml) and IL-17A (100 ng/ml) (right panels) (n = 4).

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 has demonstrated that 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

**FIGURE 6.** Increased β2 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>
<thead>
<tr>
<th>Adhesion Molecule</th>
<th>Baseline: Psoriasis/Control</th>
<th>Control: 30 min/Baseline</th>
<th>Psoriasis: 30 min/Baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1</td>
<td>1.0</td>
<td>1.3</td>
<td>1.2</td>
</tr>
<tr>
<td>CD11b</td>
<td>1.4</td>
<td>2.3</td>
<td>1.9</td>
</tr>
<tr>
<td>CD11c</td>
<td>1.5</td>
<td>2.6</td>
<td>2.2</td>
</tr>
<tr>
<td>CD18</td>
<td>1.4</td>
<td>1.8</td>
<td>1.7</td>
</tr>
<tr>
<td>VLA-4</td>
<td>1.2</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>1.2</td>
<td>1.1</td>
<td>1.1</td>
</tr>
</tbody>
</table>

*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 CD11b⁺Ly6C⁺ 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 highlights upregulation in doubles 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 ITGA-3 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 artery disease. 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.

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

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References


