Tumor-Associated Macrophages in the Cutaneous SCC Microenvironment Are Heterogeneously Activated

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Tumor-associated macrophages (TAMs) may have an important role in tumor immunity. We studied the activation state of TAMs in cutaneous SCC, the second most common human cancer. CD163 was identified as a more abundant, sensitive, and accurate marker of TAMs when compared with CD68. CD163+ TAMs produced protumoral factors, matrix metalloproteinases 9 and 11 (MMP9 and MMP11), at the gene and protein levels. Gene set enrichment analysis (GSEA) was used to evaluate M1 and M2 macrophage gene sets in the SCC genes and to identify candidate genes in order to phenotypically characterize TAMs. There was coexpression of CD163 and alternatively activated “M2” markers, CD209 and CCL18 (chemokine (C-C motif) ligand 18). There was enrichment for classically activated “M1” genes in SCC, which was confirmed in situ by colocalization of CD163 and phosphorylated STAT1 (signal transducer and activator of transcription 1), IL-23p19, IL-12/IL-23p40, and CD127. Also, a subset of TAMs in SCC was bi-activated as CD163+ cells expressed markers for both M1 and M2, shown by triple-label immunofluorescence. These data support heterogeneous activation states of TAMs in SCC, and suggest that a dynamic model of macrophage activation would be more useful to characterize TAMs.

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INTRODUCTION

Cutaneous squamous cell carcinoma (SCC) is the second most common human cancer, affecting >300,000 individuals in the United States annually (Weinberg et al., 2007; Brantsch et al., 2008). Although most cases can be treated successfully by surgical removal, certain aggressive cases can cause extensive tissue destruction and metastasize to local lymph nodes and distant organs. These aggressive cases are responsible for ~10,000 non-melanoma skin cancer deaths in the United States each year. Aggressive behavior by SCC is observed in solid organ-transplant recipients (Carucci, 2004).

Based on the potential for the host immunity to regulate tumor behavior in SCC, it is important to characterize the tumor-associated immune microenvironment.

Macrophages are one of the major populations of tumor-infiltrating leukocytes associated with solid tumors (Gordon and Taylor, 2005). Macrophages that infiltrate and surround tumor nodules are defined as tumor-associated macrophages (TAMs) (Wang et al., 2010), and different studies have shown that macrophages may either inhibit or stimulate tumor growth. Initially, TAMs were shown to participate in the early eradication of tumor cells in vitro (Romieu-Mourezu et al., 2006). However, other studies have suggested that TAMs may contribute to carcinogenesis, as there is a positive correlation between increased numbers of TAMs and poor prognosis in some human cancers (Leek et al., 1996; Bingle et al., 2002; Sica et al., 2007; Lin and Pollard, 2007; Shabo et al., 2008; El-Rouby, 2010; Nonomura et al., 2010; Steidl et al., 2010). TAMs can fail to recognize tumor antigens (Fadok et al., 1998) and may release factors that directly stimulate tumor growth and angiogenesis (Lin et al., 2006; Lin and Pollard, 2007). Furthermore, the tumor itself can create a dynamic microenvironment that can transform TAMs (Gocheva et al., 2010). Thus, TAMs in the SCC microenvironment may be associated with tumor growth.

Currently, the general classification of macrophage activation parallels the Th1/Th2 paradigm, defining classically activated (M1) and alternatively activated (M2)
cells (Mantovani et al., 2004; Mosser and Edwards, 2008). Classically activated macrophages are induced by IFN-γ and have a high capacity to present antigen. Alternatively activated macrophages are induced by the cytokine IL-4, which promotes type 2 responses. As SCCs usually progress, in association with a Th2 microenvironment and low levels of IFN-γ, it is thought that the net immune response is ineffective at suppressing tumor growth. Hence, TAMs have commonly been considered alternatively activated or strongly skewed to the M2 phenotype (Biswas et al., 2006; Martinez et al., 2009; Siveen and Kuttan, 2009; Gordon and Martinez, 2010). However, there has been renewed debate over the phenotypic activation of TAMs as the physiology of these macrophages has been shown to change over time and to demonstrate remarkable plasticity (Mosser and Edwards, 2008).

Given the importance of TAMs contributing to tumor growth, and the current conflicting state of the understanding of TAM activation, we set out to phenotypically characterize macrophages in SCC. Initially, we used a nonbiased genomic approach to guide our choice of markers to further evaluate TAMs. “M1” and “M2” activated macrophage gene sets (Fuentes-Duculan et al., 2010) were analyzed in our SCC genomic phenotype (Haider et al., 2006) using gene set enrichment analysis (GSEA) (Subramanian et al., 2005; Bluth et al., 2009). We then identified candidate genes that were expressed in the macrophage and SCC gene sets and performed immunofluorescence on SCCs versus CD163, as our constitutive macrophage marker.

Previously, we have shown that in normal skin and psoriasis, CD163 is the most useful marker of dermal macrophages (Zaba et al., 2007). We expanded on that work to characterize TAMs in human SCC. We found the following: (1) compared with CD68, CD163 was a more abundant, sensitive, and accurate marker of TAMs; (2) there was an increase in the protumoral factors matrix metalloproteinases 9 and 11 (MMP9 and MMP11) in SCC, and CD163+ TAMs produced MMP9 and MMP11; (3) there was coexpression of CD163 and alternatively activated “M2” markers, CD209 and CCL18 (chemokine (C-C motif) ligand 18); (4) there was enrichment for classically activated “M1” genes in SCC, which was confirmed in situ by colocalization of CD163 and phosphorylated STAT1 (STAT1p), IL-23p19, IL-12/IL-23p40, and CD127; and (5) a subset of TAMs in SCC was bi-activated as CD163+ cells expressed markers for both M1 and M2, shown by triple-label immunofluorescence. These data support heterogeneous activation states of TAMs in SCC, and suggest that a dynamic model of macrophage activation would be more useful to characterize TAMs. Furthermore, driving TAM activation toward a more dominant anticancer phenotype might be a potential therapeutic strategy.

RESULTS

Macrophages were more abundant in SCC compared with normal skin

Macrophages were quantified in SCC and normal skin (n = 8–18) using CD163, which we consider a reliable marker of macrophages in normal skin and psoriasis, and CD68, the widely accepted macrophage marker (Zaba et al., 2007; Bluth et al., 2009; Fuentes-Duculan et al., 2010). Representative immunohistochemistry is shown for CD163 and CD68, and cell counts of the cases are presented (Figure 1a and b). The vast majority of CD163+ and CD68+ macrophages were surrounding, rather than infiltrating, the SCC tumor nests, and both CD163+ and CD68+ macrophages were significantly increased, ~2-fold, in SCC compared with normal skin (P<0.001 for both). Additionally, using double-label immunofluorescence, CD163 colocalized with CD68 but there were CD163+ cells that did not coexpress CD68, suggesting that CD163 is a more robust and sensitive marker of macrophages in the skin than CD68 (Figure 1c). We also evaluated the coexpression of CD163 with the well-known dendritic cell (DC) marker, CD11c (Bluth et al., 2009). As we have previously shown in normal skin (Zaba et al., 2007), CD163+ cells in SCC also did not colocalize with this DC marker (Figure 1d), demonstrating that these are two distinct leukocyte populations. In contrast, CD68+ cells close to SCC tumor nests did show colocalization with CD11c (Figure 1e).

SCC TAMs expressed protumoral products in the tumor microenvironment

TAMs may produce factors that encourage tumorigenesis. We have shown that SCC TAMs produce the prolymphangiogenic factor vascular endothelial growth factor-C, which favors tumor growth and development (Moussai et al., 2011). MMPs, the enzymes that may contribute to tumor invasion by degrading the matrix surrounding tumor nodules, may also be produced by TAMs in SCC. MMP1, MMP10, and MMP13 genes have been shown to be upregulated in SCC (Haider et al., 2006). MMP9 (gelatinase B) and MMP11 (stromelysin-3) proteins correlate with increased tumor aggressiveness (Pinto et al., 2003; Buergy et al., 2009; Shah et al., 2010; Steidl et al., 2010; Zhao et al., 2010). Neither MMP9 nor MMP11 have been classified as products representative of either state of macrophage activation. We showed that there was increased gene expression by reverse transcriptase-PCR (RT-PCR) of MMP9 in SCC compared with adjacent nontumoral skin and normal skin (P = 0.07 and 0.008, respectively, Figure 2a). Similarly, MMP11 was also increased in SCC compared with adjacent nontumoral skin and normal skin (P = 0.003 and 0.025, respectively, Figure 2b). We then asked whether TAMs could be a possible source of the increased MMP9 and MMP11. There was abundant colocalization of MMP9 and MMP11 with CD163+ macrophages in SCCs compared with normal skin (Figure 2c and d). These findings suggest that TAMs secrete protumoral products in the SCC microenvironment.

SCC TAMs expressed products of alternatively activated macrophages

We evaluated expression of STAT6p based on the association of Th2 cells and the tumor microenvironment (Todaro et al., 2008; de Oliveira et al., 2009). STAT6 has an important role in signaling pathways that lead to the differentiation of Th2 cells, and STAT6p translocates to the nucleus in...
IL-4-activated cells (Takeda et al., 1996; Forbes et al., 2010). STAT6p colocalization with CD163 was abundant in the inflammatory infiltrate associated with SCC compared with normal skin (Figure 3a), suggesting the presence of IL-4 activation in TAMs.

To further evaluate the tumor microenvironment, we used M1 and M2 gene sets to correlate with the SCC genomic phenotype using GSEA (Subramanian et al., 2005; Lamb et al., 2006; Bluth et al., 2009). We have used this approach previously (Bluth et al., 2009), and have described it thoroughly in a previous publication (Suarez-Farinas et al., 2010). Fuentes-Duculan et al. (2010) recently published these sets of genes defining “M1” macrophages, induced with IFN-γ, and “M2” macrophages, induced with IL-4, compared with control. We hypothesized that there should be greater expression of M2 macrophage genes in the SCC genomic phenotype (Martinez et al., 2009; Siveen and Kuttan, 2009), defined by the SCC versus normal skin genes (Subramanian et al., 2007). However, the M2 gene set was not significantly enriched in SCC genomic phenotype, which may reflect that the M2 gene set is similarly expressed in both SCC and normal skin.

Despite the lack of enrichment of M2 genes in the SCC transcriptome, some published M2 genes (Martinez et al., 2006) were upregulated in our M2 gene set (Table 1), including CD209 (DC-SIGN) (Silleux et al., 2002; Puig-Kroger et al., 2004), CCL17 (Bonecchi et al., 1998; Katakura et al., 2004), and CCL18 (Kodelja et al., 1998; Mantovani et al., 2004; Martinez et al., 2006; Gustafsson et al., 2008; Kwan et al., 2008). By double-label immunofluorescence, CD163+ cells colocalized with CD209 and CCL18 in SCC and to a lesser extent in normal skin (Figure 3b and c). In the SCC microenvironment, we have thus shown that TAMs expressed some surface markers (CD209) and chemokines (CCL18) of M2-type macrophages. In addition and consistent with previous findings (Schutyser et al., 2005), we showed that in normal skin, macrophages at steady state are in an alternatively activated state.

Figure 1. Macrophages were more abundant in squamous cell carcinoma (SCC) compared with normal skin. Representative immunohistochemistry (×10) and cell counts of the macrophage markers (a) CD163 (with an inset of CD163+ cells at ×20) and (b) CD68, showing a significantly increased number of macrophages surrounding SCC tumor nests compared with normal skin. Each dot represents one patient. ***P<0.001. (c) CD163 (green) colocalized with CD68 (red) shown as yellow, but there were CD163+ cells that did not coexpress CD68. (d) CD163 (green) did not coexpress CD11c (red), whereas (e) some CD68+ cells (green) did coexpress CD11c (red) shown as yellow. Bar = 100 μm.
SCC TAMs expressed characteristics of classically activated macrophages in a microenvironment with type 1 activation

Although there was not any significant enrichment of M2 macrophage genes in the SCC genomic phenotype, the GSEA results did indicate that M1 macrophage gene sets were significantly enriched in the SCC genomic phenotype (Supplementary Table S1 online). Table 1 lists selected genes that were upregulated in both the M1 gene sets and the SCC versus normal skin transcriptome, indicating an M1-type macrophage activation pattern in the SCC microenvironment. It was perhaps surprising that there was such a marked M1 genomic signature in SCC. However, several recent studies have shown that IFN-γ-producing T cells can be found in SCs and other tumors (Li-Weber and Krammer, 2003; Huang et al., 2009; Kryczek et al., 2009; Koller et al., 2010).

To confirm that macrophages in the SCC microenvironment could be responsive to IFN-γ, we performed double-label immunofluorescence with CD163 and the two requisite chains of the IFN-γ receptor. The IFN-γ receptor 1 subunit (IFNγR1) that is internalized upon binding with IFN-γ (Schröder et al., 2004) was present in the majority of macrophages in SCC compared with macrophages in normal skin, which did not express this receptor (Supplementary Figure S1a online). There is a second chain of IFNγ receptor, IFNγR2, that showed a similar pattern of colocalization with CD163 + cells in SCC compared with normal skin (Supplementary Figure S1b online). CD68 + macrophages in SCC also strongly expressed IFNγR1 and IFNγR2 compared with normal skin (Supplementary Figure S1c and d online). These data indicate that SCC TAMs are capable of responding to IFN-γ. STAT1, the well-recognized IFN-γ-induced genes (Landolfo et al., 1995; Saha et al., 2010), were upregulated in the M1 gene set and SCC transcriptome (Table 1). STAT1p, the form of STAT1 that translocates to the nucleus in IFN-γ-activated cells, showed minimal expression in normal skin, but colocalized with CD163 in the juxtatumoral dermis of SCC (Figure 4a), indicating that CD163 + macrophages in SCC were indeed responding to IFN-γ.

To further classify these SCC TAMs, the expression of markers and cytokines on TAMs considered to be representative of classical/M1 macrophage activation were evaluated (Table 1) (Martinez et al., 2006). There was increased gene expression of the marker CD127 (IL-7 receptor) and the cytokine subunits, IL-23p19 and IL-12/23p40, in the M1 macrophage gene set and increased gene expression of IL-23p19 in the SCC transcriptome (Table 1). The expression of CD127 has been shown to be strongly downregulated by IL-4 (Crawley et al., 2010), which supports its role as an M1 marker. We confirmed these findings at the protein level by double-label immunofluorescence. CD163 + TAMs abundantly coexpressed CD127 and produced IL-23p19 and IL-12/IL-23p40 compared with normal skin CD163 + macrophages (Figure 4b-d). Thus, in the SCC microenvironment, TAMs express surface markers (CD127) and cytokines (IL-23 subunits) of M1-type activated macrophages.

A subset of SCC TAMs simultaneously expressed markers of both classical and alternative activation

We have shown that TAMs in the SCC environment exhibit characteristics of both classical and alternative activation. However, it was not clear if there were two separate populations of macrophages driven by each cytokine, or if one subset of macrophages was responding to both classical and alternative activation. To address this issue, we performed double-label immunofluorescence with CD163 and the two requisite chains of the IFN-γ receptor. The IFN-γ receptor 1 subunit (IFNγR1) that is internalized upon binding with IFN-γ (Schröder et al., 2004) was present in the majority of macrophages in SCC compared with macrophages in normal skin (Supplementary Figure S1a online). There is a second chain of IFNγ receptor, IFNγR2, that showed a similar pattern of colocalization with CD163 + cells in SCC compared with normal skin (Supplementary Figure S1b online). CD68 + macrophages in SCC also strongly expressed IFNγR1 and IFNγR2 compared with normal skin (Supplementary Figure S1c and d online). These data indicate that SCC TAMs are capable of responding to IFN-γ. STAT1, the well-recognized IFN-γ-induced genes (Landolfo et al., 1995; Saha et al., 2010), were upregulated in the M1 gene set and SCC transcriptome (Table 1). STAT1p, the form of STAT1 that translocates to the nucleus in IFN-γ-activated cells, showed minimal expression in normal skin, but colocalized with CD163 in the juxtatumoral dermis of SCC (Figure 4a), indicating that CD163 + macrophages in SCC were indeed responding to IFN-γ.

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cytokines simultaneously. Using triple-label immunofluorescence and confocal imaging, some CD163$^+$ cells were identified that colocalized with both STAT1p and STAT6p (shown by white arrows, Figure 5a), indicating that some SCC TAMs respond to both Th1 and Th2 signals and that there is a complex SCC tumor microenvironment of mixed Th1 and Th2 activation.

To further evaluate the TAM phenotype in this setting, we also identified a subpopulation of CD163$^+$ macrophages that coexpressed both CD127 (upregulated in M1 macrophages) and CD209 (upregulated in M2 macrophages) (shown by white arrows, Figure 5b). There were also CD163$^+$ cells that colocalized with CD127 only (yellow cells) and CD163$^+$ cells that colocalized with CD209 (dark teal cells). This suggests that macrophage activation in SCC is heterogeneous, as we found several types of TAMs: M1 macrophages responding to Th1 signals, M2 macrophages responding to Th2 signals, and bi-activated macrophages responding to both Th1 and Th2 cytokines simultaneously.

DISCUSSION

Our studies suggest that CD163 should be considered the superior marker to identify TAMs in SCC. First, CD163$^+$ TAMs were prominent and more abundant in the cutaneous SCC tumor microenvironment and significantly increased compared with macrophages in normal skin. Second, CD163 is the most sensitive marker for TAMs in SCC, as it identifies more dermal macrophages surrounding the tumor nodules than CD68. Third, CD163 is a more accurate marker of TAMs, because it did not colocalize with CD11c$^+$ DCs in SCC, compared with CD68, which showed some overlap. This is similar in psoriasis, where CD163 had the least overlap with CD11c (Fuentes-Duculan et al., 2010). In addition, although CD163 was previously considered a

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<th>Table 1. Representative upregulated genes in the M1 and M2 macrophage gene sets$^1$ and the SCC transcriptome$^2$</th>
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Abbreviations: CCL18, chemokine (C-C motif) ligand 18; Mx-1, myxovirus resistance 1; SCC, squamous cell carcinoma; STAT1, signal transducer and activator of transcription 1.

$^1$Fuentes-Duculan et al., 2010.

$^2$Haider et al., 2006.

$^3$Genes representative of M1 and M2 macrophages were selected and their fold change in the M1 and M2 gene sets and the SCC transcriptome are listed.

$^4$Genes not differentially expressed in the M1 gene set compared with control.

$^5$Genes not differentially expressed in the M2 gene set compared with control.

$^6$SCC versus normal skin transcriptome, fold change and false discovery rate (FDR) < 0.05.

$^7$HU95 chip did not include this probe.

$^8$FDR > 0.05.

Figure 3. Squamous cell carcinoma (SCC) tumor-associated macrophages (TAMs) expressed products of alternatively activated macrophages. Many CD163$^+$ cells (green) coexpressed (a) phosphorylated signal transducer and activator of transcription 6 (STAT6p; red), (b) CD209/DC-SIGN (red), and (c) chemokine (C-C motif) ligand 18 (CCL18; red) compared with normal skin. Double-positive cells appear yellow. Bar = 100 μm.
we have shown that CD163 not only identifies alternatively activated macrophages, but also classically and bi-activated macrophages in SCC. These findings suggest that future studies could benefit from using CD163 as a pan-macrophage marker in the skin.

To understand the cellular state of macrophage activation in the cutaneous SCC microenvironment, we must consider the setting in which these TAMs exist (Figure 5c). The tumor microenvironment is defined as a mixture of tumor and nontumor cells at the dynamic interface of neoplasia (van Kempen et al., 2003). Previous studies have documented an influx of IFN-γ-producing cells in the tumor microenvironment (Kryczek et al., 2009). We found a strong macrophages in SCC. These findings suggest that future studies could benefit from using CD163 as a pan-macrophage marker in the skin.

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Figure 4. SCC tumor-associated macrophages (TAMs) expressed characteristics of classically activated macrophages in a microenvironment with type 1 activation. Compared with normal skin, CD163+ cells coexpressed (a) phosphorylated signal transducer and activator of transcription 1 (STAT1p; red), (b) CD127/L7R (red), (c) IL-23p19 (red), and (d) IL-12/IL-23p40 (green). Double-positive cells appear yellow. Bar = 100 μm.

Figure 5. A subset of SCC tumor-associated macrophages (TAMs) simultaneously expressed characteristics of both classical and alternative activation. Triple-labeled confocal immunofluorescence revealed the presence of (a) CD163+ cells (blue) that simultaneously coexpressed phosphorylated signal transducer and activator of transcription 6 (STAT6p; green) and STAT1p (red) and (b) CD163+ cells (green) that simultaneously coexpressed the markers CD127 (red) and CD209 (blue) in SCC. Triple-positive cells (white) are indicated by arrows. Bar = 100 μm. (c) The proposed model of SCC macrophage polarization. Th1 and Th2 cells produce cytokines, IFN-γ and IL-4, respectively, and act on resident CD163+ macrophages to polarize these cells in several directions. IFN-γ stimulates the M1 phenotype (CD127 and IL-23), and IL-4 stimulates toward the M2 phenotype (CD209 and chemokine (C-C motif) ligand 18 (CCL18)). There is also production of mediators that are not driven by known polarizing cytokines, such as matrix metalloproteinase 9 (MMP9), MMP11, and vascular endothelial growth factor-C (VEGF-C). The overall outcome is a poly-activated TAM.
IFN-γ genomic signature in the SCC tumor microenvironment coupled with evidence of upregulated IFN-γ receptors and abundant IFN-γ activation of the infiltrating TAMs, indicating a Th1-type immune environment. However, the tumor microenvironment also has increased levels of Th2 cytokines (IL-4 and IL-10) produced by both immune and tumor cells (Lathers and Young, 2004; Pries and Wollenberg, 2006; Todaro et al., 2008; Bluth et al., 2009). In our study, there was Th2 activation shown by phosphorylated STAT6 and CCL18 coexpression by CD163+ macrophages. Thus, these data suggest that TAMs in SCC respond to a dynamic mix of Th1 and Th2 signals.

These studies show that in normal skin at steady state, there is a predominant Th2/M2 environment. In contrast, SCC TAMs demonstrate features of both M1 and M2 activation. Conventionally, strong Th1/M1 responses should prevent tumor progression (Hung et al., 1998). Furthermore, in imiquimod-treated SCC, increased levels of IFN-γ in SCC induced antitumor effects and inhibited tonic anti-inflammatory signals of IL-10 (Huang et al., 2009). However, despite the presence of a strong M1 signal, the natural history of SCC is usually tumor progression, and hence this M1 signal is not able to eradicate the tumor. The mechanism for the ineffectiveness of Th1/M1 macrophages in eradicating the tumor is not clear, but may be because of the imbalance of Th1 and Th2 cytokines and their effects on TAMs.

Our study provides insight into the heterogeneous phenotypes and functions of TAMs. The effect of M1 and M2 TAMs may be to amplify immune responses to the tumor by inducing chemotaxis and activation of infiltrating T cells. The abundant influx of macrophages into the tumor microenvironment can also help promote tumor growth by stimulating angiogenesis and tissue remodeling. The protumoral role of TAMs in SCC is supported by the production of prolymphangiogenic factors such as vascular endothelial growth factor-C (Moussai et al., 2011). Our observation that TAMs produce MMP9 and MMP11 also supports their protumoral role, as MMPs facilitate direct tumor spread and release of matrix-sequestered angiogenic factors that encourage tumor growth (Egeblad and Werb, 2002). Also, it is possible that tumors may produce factors that act on TAMs in a paracrine fashion (Balkwill and Mantovani, 2001). A positive correlation between increased numbers of macrophages and poor prognosis in various human cancers has been demonstrated (Bingle et al., 2002; Sica et al., 2006; Lin and Pollard, 2007; Shabo et al., 2008; El-Rouby, 2010; Nonomura et al., 2010; Steidl et al., 2010). It is possible that the weaker classical activation state favors production of procarcinogenic growth factors by TAMs. Based on this, the potential to induce a more dominant M1 activation state in TAMs could be pursued as a promising target for therapeutic interventions.

There has been much debate over the phenotypic activation of TAMs as the physiology of macrophages has been shown to demonstrate remarkable plasticity (Mosser and Edwards, 2008; Siveen and Kuttan, 2009). Critics of the current linear model of macrophage polarization argue that the model does not allow for the plethora of possible macrophage activation states and capabilities. Our evidence suggests that the activation state of a macrophage cannot be pigeonholed into one category, and that the linear model of macrophage activation may not encompass all the potential roles of the macrophage. Therefore, we favor the dynamic color wheel model of macrophage activation proposed by Mosser and Edwards (2008) as it accommodates the chameleon-like properties of this cell (Stout and Suttles, 2004).

### MATERIALS AND METHODS

Institutional review board approval (Weill Cornell Medical College) and informed consent was obtained before enrolling patients in this study, and the study adhered strictly to the Declaration of Helsinki Principles.

#### Samples used in the study

For immunohistochemistry and immunofluorescence, cutaneous nodular stage 1 SCC samples were obtained during Mohs microscopic surgery \((n=3-18)\). Tumors were obtained from sun-exposed regions, namely head, neck, and dorsal hands. All tumors were \(<2\,\text{cm}^{2}\) on examination and showed dermal invasion on light microscopy. Ten normal specimens were obtained via punch biopsies from non-sun-exposed areas of patients without skin cancer and normal abdominoplasty tissue.

#### Immunohistochemistry and immunofluorescence

Standard procedures were used for immunohistochemistry as described (Bluth et al., 2009). Normal skin and SCC \((n=8-18)\) were stained with macrophage markers CD163 and CD68 (antibodies in Supplementary Table S2 online) and a counterstain. Normal papillary dermis, designated as the tissue extending from the epidermal-dermal border to 100 \(\mu\text{m}\) deep to the epidermis, and SCC “juxtatumoral dermis,” defined as the dermis 100 \(\mu\text{m}\) circumferential to the tumor, were examined as previously described (Kaporis et al., 2007). Positive cells were counted using NIH IMAGE J software (Bethesda, MD), and cell counts per unit area \((\mu\text{m}^2 \times 100,000)\) were determined. Immunofluorescence stains were carried out in a standard manner (Fuentes-Duculan et al., 2010) (Supplementary Table S2 online). Images were acquired using either Zeiss Axioplan 2 widefield fluorescence microscope (Thornwood, NY) or upright confocal microscope. Dermal collagen fibers gave green autofluorescence, and antibodies conjugated with fluorochromes often gave background epidermal fluorescence. Single stain controls and isotype controls were performed for the confocal images (Supplementary Figure S2 online).

#### Gene array analysis

SCC microarray data have been previously published (Haider et al., 2006). To estimate the fold change of SCC versus normal skin, a moderated t-test available in limma package from R/Bioconductor (Seattle, WA) was used, and genes were considered significant with a fold change \(>2\) and false discovery rate \(<0.05\). GSEA was used to evaluate the enrichment of the macrophage transcriptomes in the SCC genomic phenotype (as defined by SCC vs. normal fold change by microarray) as in a previous publication by our group (Suarez-Farinás et al., 2010) using GSEA desktop application (Lamb et al., 2006; Suarez-Farinás et al., 2010). The genomic transcriptomes of
M1 and M2 macrophages derived from in vitro cytokine-stimulated macrophages have been published (Fuentes-Duculan et al., 2010). Macrophages were polarized by adding IFN-γ, IL-4, tumor necrosis factor-α, lipopolysaccharide, and lipopolysaccharide plus IFN-γ, and then compared with control macrophages. There were 585 upregulated and 334 downregulated probes in the M1 macrophages, respectively, and 132 upregulated and 29 downregulated probes in the M2 macrophages, respectively (Fuentes-Duculan et al., 2010).

RT-PCR
RNA was extracted from paired SCC and nontumoral skin samples meeting the above inclusion criteria (n = 15) and normal skin from healthy volunteers (n = 10), using the RNeasy Mini KIT (Qiagen, Valencia, CA). RT-PCR was performed as previously described (Chamian et al., 2005; Fuentes-Duculan et al., 2010). The PCR was performed in two batches. The first group was 10 paired SCC and nontumoral skin, and 2 normals, and the second group was 5 paired SCC samples (described in Moussai et al., 2011) and 8 normal skin RNA. The primers for MMP9 and MMP11 were from Applied Biosystems, Foster City, CA (Hs00957562_m1 and Hs00171829_m1, respectively), normalized to Human Acidic Ribosomal Protein housekeeping gene. As samples were obtained at two different time points, the log2 data were adjusted using a linear model to account for the batch effect.

Statistics
Cell counts were analyzed using Mann-Whitney U-test, and P-values reported. Logarithmic RT-PCR data were analyzed using paired t-test for SCC versus nontumoral skin and unpaired t-test to compare with normal skin control.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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