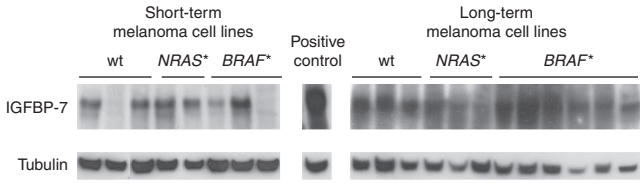

Figure 2. Lack of correlation between *NRAS/BRAF* mutational status and IGFBP7 expression in melanoma cell lines. The *NRAS/BRAF* status of cell lines was determined by sequencing. Lysates from these short- and long-term cultured melanoma cell lines were subsequently evaluated using western blot analysis for IGFBP7 expression.

IGFBP7 of *BRAF*-mutated melanoma cells triggered their apoptosis both *in vitro* and *in vivo* in a xenotransplantation model. Similarly, reactivation of IGFBP7 by DNA demethylation inhibits colon cancer cell growth *in vitro* (Lin *et al.*, 2008). With this therapeutic implication in mind, we further scrutinized the expression of IGFBP7 in melanoma. To this end, we stained formalin-fixed tissues obtained from 41 primary tumors and 16 metastases for IGFBP7 expression. Unlike Wajapeyee and colleagues, however, we did not detect a clear demarcation of IGFBP7 expression between *BRAF* wt (wild type) and mutated melanoma lesions (Figure 1). Indeed, although the frequency of IGFBP7-expressing cells is lower in *BRAF*-mutated melanoma lesions overall, the whole diversity of IGFBP7 expression from absent to present in 100% of tumor cells was observed in both wt and *BRAF*-mutated lesions ($P=0.1609$; Mann–Whitney test). Accordingly, western blot analysis of short- and long-term cultured melanoma cell lines did not show any correlation between IGFBP7 protein expression and *BRAF* status either (Figure 2). Heterogeneous IGFBP7 expression could still be in accordance with the reported stringent IGFBP7/BRAF correlation, assuming a corresponding heterogeneity in the *BRAF* status. However, the homogeneous IGFBP7 expression in some of the *BRAF*-mutated tumors, as well as the lack of correlation for the cell lines, with one of them being hemizygote for the V600E BRAF mutation, argues against an obligatory downregulation of IGFBP7 expression in *BRAF*-mutated melanoma cells. Rather, it seems that loss of IGFBP7 expression is not the only way to overcome MAPK pathway–induced senescence. In this regard, dysregulation of the other 16 candidates detected by Wajapeyee *et al.* (2008), including BNIP3L, FOXA, and NF2, may be alternative mediators for overcoming senescence. Thus, detailed studies scrutinizing different *modi operandi* by which melanoma cells overcome BRAF-induced senescence are needed before the potential of IGFBP7 substitution for treatment of melanoma can be estimated.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Indoleamine 2,3-Dioxygenase$^+$ Cells Correspond to the BDCA2$^+$ Plasmacytoid Dendritic Cells in Human Melanoma Sentinel Nodes

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TO THE EDITOR

Dendritic cells (DCs) have crucial roles in driving primary immune responses toward immunity or tolerance. While immature DCs prompt tolerance in peripheral tissues, mature DCs drive immunity in lymph nodes (Banchereau *et al.*, 2000). A particular subset of DC, however, is thought to have the ability to induce tolerance regardless of the maturation state (Gilliet and Liu, 2002).

Abbreviations: DC, dendritic cell; IDO, indoleamine 2,3-dioxygenase; pDC, plasmacytoid DC; SLN, sentinel lymph node; TLR, toll-like receptor

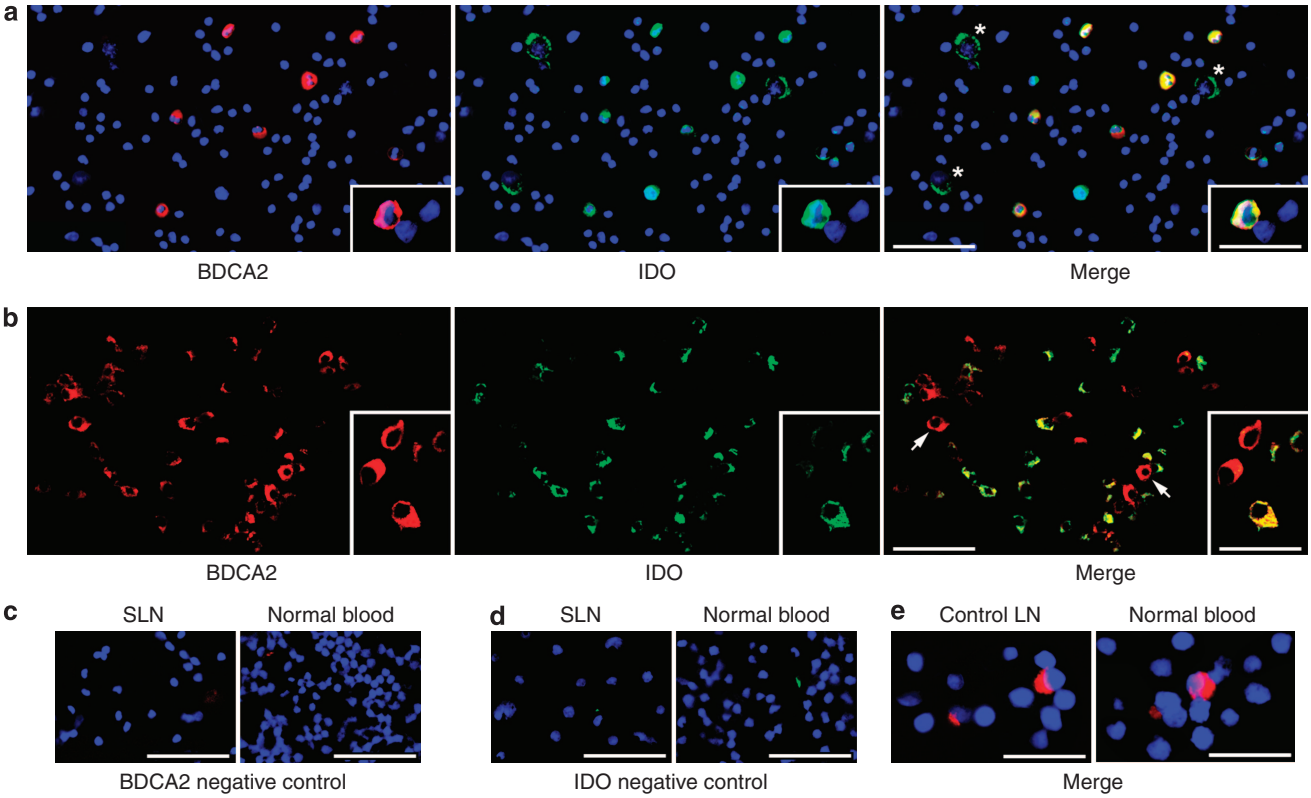

Figure 1. Sentinel lymph node (SLN) BDCA2$^+$ plasmacytoid dendritic cells (pDCs) express indoleamine 2,3-dioxygenase (IDO). Immunofluorescence analyses of pDC in human melanoma SLN ($n = 15$: 4 positive for metastasis and 11 negative). Labeling was performed with mouse anti-human BDCA2, to identify pDC (revealed with goat anti-mouse Alexa Fluor 594 conjugated; red) and with rabbit anti-human IDO (revealed with goat anti-rabbit FITC conjugated; green). Nuclei were labeled with Hoechst 33342 (blue). (**a**) Immunofluorescence on cells from a metastatic SLN is shown. BDCA2$^+$ pDC coexpressed IDO (yellow), although at a various degree of intensity. A subset of BDCA2$^-$/IDO$^+$ large cells were also identified (indicated by asterisks). Inset, high magnification of a BDCA2$^+$/IDO$^+$ pDC. (**b**) Immunofluorescence on frozen section from a metastatic SLN is shown. The majority of BDCA2$^+$ cells coexpressed IDO (yellow), with only a few being IDO$^-$ (indicated by arrows). Inset, high magnification of BDCA2$^+$/DO$^+$ (yellow) and BDCA2$^+$/IDO$^-$ (red) pDC. (**c–d**) Isotype controls for anti-BDCA2 Ab (mouse IgG1) and anti-IDO Ab (rabbit IgG), in SLN and normal blood cytospins, respectively. No specific stainings were detected. (**e**) Immunofluorescence on control LN and normal blood cytospins are shown. Control LN and blood BDCA2$^+$ pDC did not express IDO (scale bars: **a, b**, 75 μm and 30 μm in the insets; **c, d**, 75 μm; **e**, 30 μm).

Munn *et al.* (2002) have described a subset of CD123$^+$/CCR6$^+$ DC in sentinel lymph nodes (SLN) of cancer patients with regulatory function. These cells express high levels of indoleamine 2,3-dioxygenase (IDO), the tryptophan-catabolizing enzyme, which is emerging as a master regulator of tolerance (Munn *et al.*, 1998; Liu *et al.*, 2006).

Indoleamine 2,3-dioxygenase$^+$ cells are present in SLN of patients with melanoma, breast, colon, lung, and pancreatic cancer (Munn and Mellor, 2007). A high number of IDO$^+$ cells occur in 45% of melanoma SLN, with or without metastasis. Importantly, this accumulation correlates with poor outcome (Lee *et al.*, 2003) strongly suggesting a tolerance role for IDO$^+$ cells. They are located in perisinusoidal regions of nodes, surrounding high endothelial venules; they exhibit monocytoid or plasmacytoid morphology and, in metastatic SLN, form clusters around melanoma nests. Therefore, IDO expression seems to be a crucial, tolerogenic mechanism in cancer immunity (Munn and Mellor, 2007). Consistently, IDO overexpression in tumor cells protects them from immune-mediated rejection, whereas the IDO inhibitor 1-methyl tryptophan can revert to tumor immune tolerance (Muller *et al.*, 2005; Hou *et al.*, 2007).

Plasmacytoid DCs (pDCs) are the main source of the antiviral/antitumor Type I IFN (Siegal *et al.*, 1999; Liu, 2001). Paradoxically, *in vitro* studies show an active role of pDC in tolerance induction (Gilliet and Liu, 2002; Ito *et al.*, 2007). Whether they have any role in cancer immunology has not been established. Yet, pDC, identified by the expression of the specific marker BDCA2 (Dzionek *et al.*, 2001), are present in SLN, with high frequency in those bearing metastasis (Gerlini *et al.*, 2007). Evidence that these pDC show an inactive immunophenotype and do not produce IFN-α hints that they favor tumor tolerance in humans (Gerlini *et al.*, 2007). In keeping with a role for both pDC and IDO in suppression of tumor immunity, IDO is expressed by a subset of mouse pDC in melanoma

tumor-draining lymph nodes. These cells prompt antigen-specific anergy, strengthening the hypothesis that IDO$^+$ pDC are indeed tolerogenic (Munn *et al.*, 2004).

The existence and function of human IDO$^+$ pDC is as yet unknown. This is probably because the antibodies available against BDCA2 are not suitable for paraffin-embedded archive material. To understand whether IDO$^+$ DC correspond to pDC, we took advantage of a recently described method for phenotypic analysis of cells from fresh and frozen human SLN, which does not interfere with pathological diagnosis (Vuylsteke *et al.*, 2002; Gerlini *et al.*, 2007). The study was conducted according to the Declaration of Helsinki Principles, and the Institutional Ethics Committee approved all described studies.

Samples were double stained with mouse anti-human BDCA2 and rabbit anti-human IDO. Variable numbers of BDCA2$^+$ pDC were found in all the 15 SLN analyzed by flow cytometry ($0.45 \pm 0.11\%$, mean $\pm$ SD), and the majority of them coexpressed IDO (72.4 ± 12.4 %), as assessed by immunofluorescence (Figure 1a and b). Notably, a subset of BDCA$^-$/IDO$^+$ large cells were also identified (Figure 1a). This finding suggests that other cells express IDO in SLN. As pDC accumulate in metastatic SLN and typically form clusters, frozen sections of metastatic SLN were investigated. Numerous BDCA2$^+$ IDO$^+$ pDC were observed, with some IDO-negative pDC scattered throughout the tissue ($31.4 \pm 9.5\%$) (Figure 1b). Functionally, BDCA2$^+$ pDC did not produce IFN-α (data not shown, Gerlini *et al.*, 2007). Importantly, control LN and blood pDC were IDO negative (Figure 1e and see Supplementary material for details), suggesting that IDO expression may be specific to tumor SLN.

The role of pDC in cancer immunology is still a matter of debate, as these cells able to produce Type I IFN (Siegal *et al.*, 1999; Liu, 2001), as well as promote tolerance (Munn *et al.*, 2004; Gerlini *et al.*, 2007). By showing that most of pDC express the immunosuppressive IDO in SLN of patients with melanoma, our findings strongly suggest that pDC have a tolerogenic role in human cancer. Recently, human pDC have been shown to upregulate IDO in response to HIV infection (Boasso *et al.*, 2007). It is possible, therefore, that metastatic melanoma exploits this mechanism to repress immune effector functions in SLN (Cochran *et al.*, 2006). Taken together, these findings indicate that the identification of IDO$^+$ cells as the classical BDCA2$^+$ pDC of SLN is not only a matter of immunophenotypic detail but has remarkable immunopathological and therapeutical significance.

Currently, different pharmacological strategies have been adopted to circumvent tumor tolerance in SLN. Toll-like receptor (TLR) agonists, such as the TLR9 activator PF-3512676, promotes DC-dependent proliferation of melanoma-specific CD8$^+$ T cells as well as effector natural killer cell responses (Molenkamp *et al.*, 2007, 2008). Similarly, the TLR7 agonist imiquimod reverts functional defect in pDC of SLN, and boosts tumor-specific immune responses in melanoma patients (Molenkamp *et al.*, 2007; Adams *et al.*, 2008). We claim that abrogating the tolerogenic effects of IDO by means of chemicals such as 1-methyl tryptophan (Hou *et al.*, 2007) plus the concomitant use of TLR7/9 agonists might be an innovative pharmacological strategy to revert tumor tolerance in SLN.

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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Somatic Mutation of Epidermal Growth Factor Receptor in a Small Subset of Cutaneous Squamous Cell Carcinoma

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TO THE EDITOR

Non-melanoma skin cancer is the most prevalent cancer in man. For reasons that are unclear non-melanoma skin cancer is dramatically increased in organ transplant recipients (OTRs) compared with immunocompetent patients (non-OTRs) (Euvrard *et al.*, 2003). Increased risk of metastasis and the presence of multiple tumors are a significant source of morbidity in OTRs. These patients present with different types of non-melanoma skin cancer with squamous cell carcinoma (SCC) and keratoacanthoma (KA) particularly prevalent. The genetic aberrations driving these cancers in OTRs and non-OTRs are poorly understood.

Receptor tyrosine kinases are frequently activated by somatic mutations and/or amplification in human cancers, particularly in epithelial tumors. To determine whether receptor tyrosine kinases are mutated in SCC, we searched the literature to identify receptor tyrosine kinases that have a role in epidermal homeostasis and thus could be candidate oncogenes in squamous lesions. We chose to analyze *epidermal growth factor receptor* (*EGFR*) that is highly expressed in a small subset of metastatic cutaneous SCCs (Bauknecht *et al.*, 1985; Shimizu *et al.*, 2001; Maubec *et al.*, 2005); *fibroblast growth factor receptor 3* (*FGFR3*) that is mutated in familial acanthosis nigricans and Crouzon's syndrome, a type of craniosynostosis (Berk *et al.*, 2007) and induces acanthosis and benign tumors in transgenic mice (Logie *et al.*, 2005); and *fibroblast growth factor receptor 2* (*FGFR2*), which is also mutated in Crouzon's syndrome and in this disease is associated with acanthosis nigricans (Meyers *et al.*, 1995). We included the *insulin-like growth factor receptor 1* (IGF1R) mice lacking this receptor have hypoplastic skin (Liu *et al.*, 1993; De Moerlooze *et al.*, 2000) and *MET*, the receptor for the ligand hepatocyte growth factor. Mice overexpressing the MET receptor exhibit an enhanced number of hair follicles and accelerated hair follicle morphogenesis (Lindner *et al.*, 2000), a feature associated with cyclosporine use in OTRs. Finally, we assessed ERBB2, which induces SCCs when targeted to mouse skin (Kiguchi *et al.*, 2000).

We determined the mutation status of the kinase domains of *EGFR*, *IGF1R*, *MET* and *ERBB2*, and the regions of *FGFR2* and *FGFR3* that are mutated in Crouzon's syndrome in a cohort of 95 tumors that consisted of 70 SCCs and 25 KAs from 55 OTR and 40 non-OTR tumors; not every tumor was analyzed for every gene. Genomic DNA was extracted from archival formalin-fixed paraffin-embedded samples and amplified with M13 sequence-tailed primers (Supplementary Table 1).

Mutations were found in *EGFR*, *FGFR2*, and *FGFR3* but not in *ERRB2*, *MET*, and *IGF receptor 1* (Table 1). The somatic nature of the mutations was confirmed by sequencing the adjacent normal skin in all three cases in which mutations were found. *EGFR* was mutated in 1 of 40 (2.5%) SCCs, a frequency not dissimilar to that detected in head and neck SCCs (7.3%) (Willmore-Payne *et al.*, 2006). The particular Y727H mutation we found in exon 18 of EGFR has been observed in SCC of the lung (Pallis *et al.*, 2007). In addition to mutational activation, amplification of wild-type *EGFR* can drive tumorigenesis in a variety of cancers and in head and neck SCC cell lines (Weichselbaum *et al.*, 1989). In a dataset of array-based comparative

Abbreviations: FGFR, fibroblast growth factor receptor; KA, keratoacanthoma; OTR, organ transplant recipient; RTK, receptor tyrosine kinase; SCC, squamous cell carcinoma