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Commentary

Imiquimod is a TLR agonist that is used as an antitumor agent, mainly against skin tumors. Its clinical benefits are well described in several studies; however, the mechanisms behind its antitumor effects are not completely understood. In this issue of the *JCI*, Drobits and colleagues demonstrate that topical application of imiquimod suppresses cutaneous melanoma by TLR7-dependent recruitment and transformation of plasmacytoid dendritic cells into killer cells; this occurs independently of conventional adaptive immune mechanisms.

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Plasmacytoid dendritic cells lead the charge against tumors

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Imiquimod is a TLR agonist that is used as an antitumor agent, mainly against skin tumors. Its clinical benefits are well described in several studies; however, the mechanisms behind its antitumor effects are not completely understood. In this issue of the *JCI*, Drobits and colleagues demonstrate that topical application of imiquimod suppresses cutaneous melanoma by TLR7-dependent recruitment and transformation of plasmacytoid dendritic cells into killer cells; this occurs independently of conventional adaptive immune mechanisms.

Imiquimod is a synthetic imidazoquinoline with demonstrable antiviral and antitumor properties. It is formulated as a 5% cream and has been approved to treat certain types of actinic keratoses, which are premalignant, flat, scaly growths on the skin caused by too much sun exposure; some primary skin malignancies, including basal cell carcinoma (BCC) and squamous cell carcinoma (1); and external genital warts. The efficacy of imiquimod as a treatment for BCC is impressive: complete histological clearance is achieved in almost 80% of the cases (2). Imiquimod has also been used off-label to treat cutaneous melanoma and locally recurrent mucosal melanoma (3), although it is a less effective treatment for this primary skin malignancy than it is for BCC (4).

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Imiquimod modulates diverse immune responses through TLR7 ligation

The antitumor effect of imiquimod is multifactorial. It has direct antiangiogenic (5) and caspase-mediated proapoptotic activity (6). However, it also acts as an "immune response modifier," modulating the function of immune cells, especially antigen-presenting cells such as conventional dendritic cells (cDCs), plasmacytoid dendritic cells (pDCs), monocytes, and macrophages. The effects of imiquimod on immune cells are mediated via activating signals initiated by its binding to TLR7 and, in some instances, to TLR8 (4).

TLRs comprise a family of conserved, germline–encoded pattern recognition receptors that recognize diverse molecules from pathogens and are essential for host defense (7). Upon ligand binding, TLRs recruit an adaptor molecule, most frequently MyD88, and promote activation of signaling molecules and pathways (including the NF-κB and MAPK pathways) that lead to the production of proinflammatory cytokines (e.g., TNF and IL-12). In pDCs, activation of the transcription factor IRF7 after TLR7 ligation leads to MyD88-dependent production of type I IFNs and upregulation of TNF-related apoptosis-inducing ligand (TRAIL) and granzyme B (7), which confer on the pDCs a death-inducing effector function. IL-1β and IL-18 are also released through TLR-independent inflammasome activation (7). Imiquimod can directly (via a TLR) or indirectly (via the type I IFNs derived from imiquimod-stimulated pDCs) stimulate NK cells, $CD8^+$ T cells, $CD4^+$ T cells, and B cells. Agonists of TLRs, including imiquimod, can also reverse $CD4^+$ Treg function in a TLR8 signaling–dependent, DC-independent manner (8).

As a result of their multifactorial antitumor effects, TLR agonists such as imiquimod are being widely tested as adjuvant therapies in cancer patients. Their administration has been associated with intratumoral infiltration of multiple subsets of DCs, and, when delivered in combination with tumor-associated antigens in humans, they have resulted in delayed time to tumor recurrence (4). However, TLR-activated cDCs and pDCs can also induce and/or recruit immune suppressive Tregs in vitro and in vivo, and high levels of pDCs in the tumor microenvironment are associated with poor prognosis (9), indicating that we are far from understand-

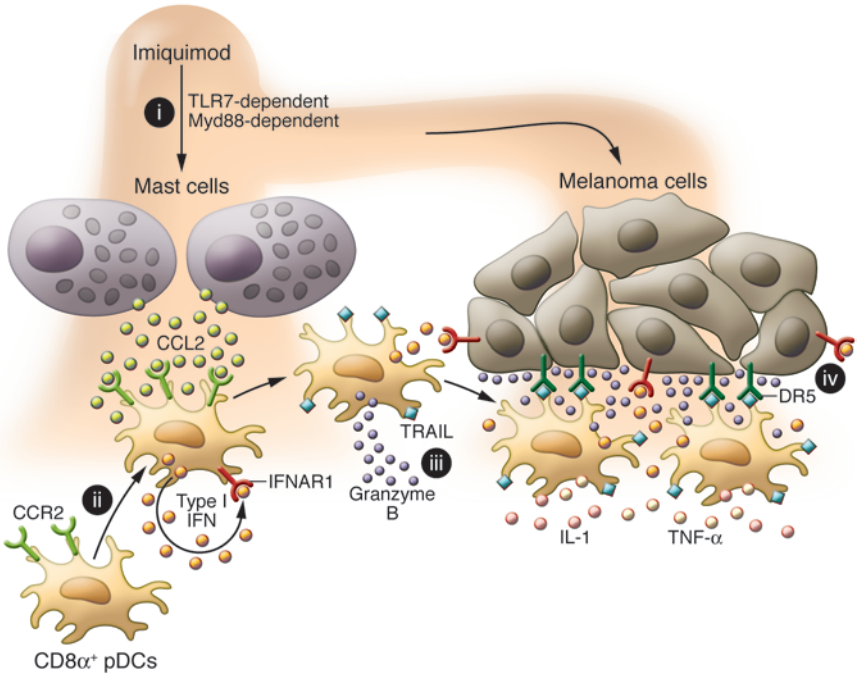

Figure 1
Mechanism of imiquimod-mediated tumor cell killing by pDCs. (i) Skin application of imiquimod acts on mast cells through TLR7, inducing the secretion of CCL2. (ii) CD8α+ pDCs migrate to the skin toward CCL2 while at the same time being exposed to imiquimod. Imiquimod-stimulated pDCs produce high levels of type I IFNs, which have several effects. First (iii), in a paracrine loop, type I IFNs induce pDCs to kill tumor cells through upregulation of expression and/or secretion of cytolytic molecules such as TRAIL and granzyme B. Second (iv), type I IFNs induce on melanoma cells the expression of the receptor for TRAIL (DR5), which makes them susceptible to killing by TRAIL-expressing pDCs.

ing how TLR agonists such as imiquimod modulate immune cell function and how to appropriately utilize them for the treatment of cancer.

In this issue of the *JCI*, Drobits and colleagues shed some light on these matters by examining the consequences of topical imiquimod application in an orthotopic mouse model of melanoma (10). The data generated identify pDCs as leaders of the antitumor response initiated by imiquimod.

Imiquimod clears melanoma through pDC-dependent innate immune mechanisms

Drobits and colleagues found that imiquimod rapidly stimulated resident dermal mast cells to produce the chemokine CCL2 and that this promoted the migration of imiquimod-activated pDCs expressing CCR2, the CCL2 receptor, into the skin (ref. 10 and Figure 1). Importantly, the recruited pDCs were fully equipped to produce type I IFNs, which matured the pDCs into cytolytic cells and triggered the upregulation of specific receptors on melanoma cells that made them susceptible to the cytolytic effects of pDCs. Most significantly, the authors showed that imiquimod-mediated suppression of tumor growth was a direct consequence of the IFN-dependent cytolytic function of pDCs rather than direct apoptotic effects of type I IFNs and that it was independent of other innate or adaptive immune responses.

CCL2, is it bad or is it good?

The critical role played by dermal mast cells in the imiquimod-induced antitumor response defined by Drobits et al. (10) is novel. Mast cells, predominantly localized in the skin and mucosal surfaces, are known effectors of innate immunity, able to induce inflammatory reactions within minutes. They express TLR7 and release proinflammatory cytokines upon its ligation (11). Using *Ccl2*$^{-/-}$ mice, Drobits et al. showed that mast cell–associated CCL2 production was required for recruitment of pDCs into cutaneous tumors (10). Interestingly, the CCL2-mediated antitumor effects were due to its capacity to "condition" type I IFN–producing pDCs to migrate to inflamed skin and execute their killing function. Tumor cells have been shown to produce CCL2 (a capacity not evaluated in the present study), and this has not always been linked with a favorable outcome (12). Indeed, CCL2 produced by tumor cells or tumor stroma has been associated with tumor progression in mice as a result of its ability to recruit inflammatory CCR2-expressing monocytes that enhance the subsequent extravasation of the tumor cells through effects on endothelial cells (12). These mouse data, together with an observed clinical association between CCL2 overexpression in human cancers and poor prognosis (12), and the fact that CCL2-specific neutralizing antibodies inhibit human melanoma xenograft progression (13) have instead supported the development of therapeutic approaches that neutralize CCL2 activity. It seems, therefore, that the stimulus, source, and mechanisms of CCL2 action might be pivotal in the final outcome of an antitumor response.

Imiquimod's dual role: controlling "attraction" and "activation"

The pattern of pDC migration reflects a complex interchange between chemokine receptor expression by pDCs and chemokine secretion in lymph nodes and inflamed tissues (14). In blood, pDCs, either constitutively or following activation, express CCR7 and respond to CCL21 in high endothelial venules, allowing them to home to lymph nodes. They may then traffic to peripheral sites such as inflamed skin by acquiring the ability to respond to CCL2 and other locally produced chemokines. This pattern of pDC migration is consistent with the concept that pDCs become activated in a sequential fashion. Besides CCL2, imiquimod induces the production of the chemokine ligands for CXCR3 (15) and the skin-homing chemokine CCL20 (14). A recent study suggested that upregulation of CCR6 and CCR10 (the receptors for CCL20 and CCL27/28) on pDCs is crucial for imiquimod-dependent pDC migration to the skin (14), although the relevance of CCL20 and whether CCR6-expressing pDCs acquire a killer phenotype was not investigated in the model studied by Drobits et al. Tumors including melanomas also produce chemokines that attract pDCs (16), but these may not activate their

antitumor potential. The data generated by Drobits and colleagues (10), however, indicates for the first time that imiquimod has the dual advantage of conditioning skin resident and attracting migrating pDCs to produce type I IFNs and cytolytic molecules while at the same time regulating the production of chemokine gradients in the skin that control pDC trafficking.

The work of Drobits et al. showing that pDCs recruited into imiquimod-treated skin acquire cytolytic capacity against tumor cells (10) is in line with other reports suggesting an emerging role for pDCs as antitumor effector cells (15, 17, 18). Chaperot et al. first showed that pDCs could directly kill melanoma cell lines in a TLR7-dependent manner (17). This effect was later described in HIV infection, where TLR7-activated pDCs were found to kill virus-exposed $CD4^+$ T cells (19). In skin tumors such as BCC there is evidence of pDC activation, local secretion of IFN-α (18), and pDC expression of TRAIL at the imiquimod-treated site (15). Drobits et al. reaffirmed the imiquimod-derived pDC killer phenotype (10) and demonstrated, using knockout mice, that it is dependent on TLR7, MyD88, and the receptor for type I IFN (IFNAR1). It was previously described how type I IFNs are essential for upregulation of the cytolytic molecules TRAIL and granzyme B on pDCs and the TRAIL receptor DR5 on tumor cells (7). Furthermore, Drobits and colleagues demonstrated that type I IFNs also control the initial production of CCL2 after imiquimod application, as *Ifnar1*$^{-/-}$ mice failed to produce CCL2 or to recruit pDCs into the dermis, suggesting that type I IFNs produced by the few resident dermal pDCs may be required to initiate the CCL2 gradient in the dermis.

Is tumor clearance reliant on pDCs alone?

Together, the data generated by Drobits et al. (10) led them to envision a two-phase response during imiquimod therapy that begins with fully competent pDC recruitment to the tumor area within 3 days, followed by a cascade of type I IFN–dependent mechanisms that contribute to enhancing the antitumor effect (Figure 1). This sustained antitumor effect was shown to occur without the contribution of other immune cells, as early depletion of NK or T cells did not affect tumor regression, giving pDCs a unique and individual role as tumor-killing cells at this stage. However, we believe that it is unlikely that pDCs alone maintain melanoma cells in "equilibrium" in the long term. Although the role of other immune cells was not tested at later time points, it is known that tumor-derived chemokines, in particular CCL2, can attract T cells, while type I IFNs can recruit T cells, B cells, and $CD8\alpha^+$ DCs into the tumor (20). Along those lines, imiquimod-treated skin lesions and tumors show a prominent inflammatory cell infiltrate (including $CD8^+$ T cells, NK cells, and B cells; refs. 15, 21) that is sometimes associated with tumor regression. Moreover, direct injection of TLR9 agonists into melanomas has resulted in pDC-mediated NK cell activation and NK cell–mediated tumor cell death, resulting in cross-presentation of tumor antigens to T cells (22). Imiquimod-treated tumors also contain a decreased percentage of Tregs, both those that express $Foxp3^+$ and those that produce IL-10 and TGF-β (23). In fact, Drobits et al. found that when $CD4^+$ T cells were depleted in their model, tumor growth decreased (10), and this could perhaps be explained by a loss of Tregs. Together, these studies suggest that strong adaptive immune responses will ultimately be necessary to contain local tumor growth, metastatic disease, and local recurrences, and thus far, imiquimod treatment has resulted only in limited regression or stabilization of cutaneous melanoma metastasis (24).

Final remarks

In summary, TLR agonists such as imiquimod possess important properties that can be exploited for immunotherapy against tumors such as melanoma. As vaccine adjuvants, they are showing promise in the clinic against various cancers (4), even when injected directly into the tumor (25). However, further studies, especially those examining their effects in combination with radiation, chemotherapy, new biologics (e.g., antibodies specific for CTLA-4 or PD-1), and/or signaling pathway inhibitors (e.g., inhibitors of BRAF or MEK), are necessary in order to determine the most effective ways to improve their antitumor activity. Moreover, we cannot forget that some tumor cells express TLRs and this may eventually modulate the outcome of the antitumor response elicited by TLR agonists such as imiquimod (4). Finally, considering the new data generated by Drobits and colleagues, attention needs to be given to how efficiently chemokines, which attract or recruit multiple types of antitumor cells into tumors, are elicited to optimize the outcome of TLR agonist therapy. Addition of chemokines and/or application of combinations of TLR agonists that elicit relevant chemokines directly into tumors may improve the fight against resistant skin tumors.

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Genetic instability in neural stem cells: an inconvenient truth?

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The evolutionary struggles from which mutants arise have been documented in almost every living system. In this issue of the *JCI*, Varela and colleagues extend this list of systems to include neural derivatives of human embryonic stem cells, which they show exhibit a repeated gain of material from chromosome 1q. Although this raises safety issues for therapeutic use of such cells, the frequent observation of a particular change may direct screening strategies for detection and removal of these unwanted cellular variants.

The excitement surrounding human ES cell research is indicative of the potential that these cells hold for regenerative therapy. However, realizing this potential requires efficient derivation of the necessary cell type and also a guarantee that the differentiated product poses no threat to the patient. With respect to these prerequisites, robust differentiation protocols have been developed for the generation of several cell types, in particular those of the neural lineage. For example, a culture of approximately 90% neural progenitors can be generated from human ES cells by simply adding dorsomorphin (a selective small molecule inhibitor of bone morphogenetic protein signaling) to a basal growth media (1). Furthermore, the fact that the ES cell–derived neurons possess the characteristics of those found in vivo has prompted clinical trials assessing their potential for therapy. Geron Corp. was among the first to test the waters with the intention of creating tissue to repair spinal cord injury, although financial issues have brought a premature end to this trial.

The issue of transplant safety, however, remains a potential stumbling block. Unlike differentiation efficiency, safety is a difficult parameter to quantify, and our knowledge is limited by the lack of data from human recipients. As a result, the stem cell field seems to have drawn a direct correlation between risk and mutation, so that any tissue whose genomic integrity has been compromised is not considered suitable for therapy. Human ES cell research has had to contend with this issue since 2004, when karyotypic changes were first reported in cultured human ES cells (2), although changes in tissue-derived cells have only been sporadically reported. With this in mind, the impact of the report in this issue of the *JCI* by Varela and colleagues that human ES cell–derived neural stem cells (NSCs) recurrently acquire genetic changes (3) must be considered.

Nonrandom mutation in NSCs: chromosome 1q jumps out

The key message from the work of Varela et al. is that, regardless of origin or genetic background, NSCs derived from pluripotent human cells frequently acquire amplification of material from chromosome 1q (3). Indeed, all NSC lines tested by the authors showed chromosome 1q amplification, invariably as part of an unbalanced translocation. This abnormality was not seen in any of the human ES cell or induced pluripotent stem (iPS) cell lines from which the NSC lines were derived, which implies it occurred specifically during the culture of NSCs. Regardless of recipient chromosome, the variant cells went on to dominate the culture, which suggests that these cells have a growth advantage over their diploid neighbors. Furthermore, the authors' data suggest that the amplification of chromosome 1q was associated with loss of senescence in these cells. This link between mutation and immortality raises an uncomfortable comparison with cancer, highlighted by the fact that the chromosome 1q jumping translocation detected by Varela and colleagues (3) has previously been observed in a number of malignancies, including pediatric brain tumors (4, 5). However, it should be noted that the abnormal NSCs were unable to form tumors in mouse models.

Mutation and selection in NSCs

Although the chromosomal changes detected by Varela and colleagues are considered undesirable in a therapeutic setting, the inconvenient truth is that they may be inescapable, and merely demonstrate the fundamental process of natural selection. Evolutionary influences act in all living systems, with random mutation creating variants better suited to growth in

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