Innate sensing of microbial products promotes wound-induced skin cancer

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The association between tissue damage, chronic inflammation and cancer is well known. However, the underlying mechanisms are unclear. Here we characterize a mouse model in which constitutive epidermal extracellular-signal-regulated kinase-MAP-kinase signalling results in epidermal inflammation, and skin wounding induces tumours. We show that tumour incidence correlates with wound size and inflammatory infiltrate. Ablation of tumour necrosis factor receptor (TNFR)-1/-2, Myeloid Differentiation primary response gene 88 or Toll-like receptor (TLR)-5, the bacterial flagellin receptor, but not other innate immune sensors, in radiosensitive leukocytes protects against tumour formation. Antibiotic treatment inhibits, whereas injection of flagellin induces, tumours in a TLR-5-dependent manner. TLR-5 is also involved in chemical-induced skin carcinogenesis in wild-type mice. Leukocytic TLR-5 signalling mediates upregulation of the alarmin HMGB1 (High Mobility Group Box 1) in wound-induced papillomas. HMGB1 is elevated in tumours of patients with Recessive Dystrophic Epidermolysis Bullosa, a disease characterized by chronic skin damage. We conclude that in our experimental model the combination of bacteria, chronic inflammation and wounding cooperate to trigger skin cancer.
The association between skin wounding, inflammation and cancer is well established. For example, Marjolin’s ulcers are aggressive squamous cell carcinomas (SCCs) that specifically develop on areas of previous skin trauma. Keloid scarring is a consequence of aberrant wound healing and is also described as benign fibrotic tumour formation. In addition, malignancies at wound sites are often overlooked in chronic ulcers in diabetic and elderly patients. Another context in which the association between skin wounding and cancer is well established is Recessive Dystrophic Epidermolysis Bullosa (RDEB). This inherited skin blistering disease is characterized by repetitive cycles of wounding and repair and is linked with a high incidence of SCC formation. Almost 100% of RDEB patients will develop at least one SCC. However, despite the clear link between skin damage and cancer, little is known about the underlying mechanisms.

We previously described a mouse model of wound-induced skin cancer that mimics key features of human hyperproliferative skin conditions. Wounded human skin and psoriatic lesions are characterized by misexpression of β1 integrin extracellular matrix receptors in the differentiating epidermal cell layers, and consequent upregulation of extracellular signal-regulated kinase-MAP-kinase signalling. When this is modelled in transgenic mice by expression of constitutively active MAP-kinase kinase 1 under the control of the involucrin promoter (InvEE mice), there is chronic skin inflammation and epidermal hyperproliferation, and mice develop benign tumours (papillomas) on wounding. We previously identified a pro-tumorigenic role for macrophages and peripheral γδ T cells in this model.

Here we set out to identify the molecular signalling events that underlie the link between chronic inflammation, tissue damage and skin cancer. We have found a previously unknown role for Toll-like receptor (TLR)-5, the receptor for bacterial flagellin, in skin tumour formation, both in the InvEE mouse model and in wild-type (WT) mice treated with chemical carcinogens. We further show that TLR-5 plays a role in upregulation of the alarmin HMGB1 (High Mobility Group Box 1) in wound-induced mouse tumours and demonstrate that HMGB1 is also elevated in tumours of RDEB patients.

**Results**

**Effect of wound size and immune infiltrate on tumorigenesis.**

To test whether wound size, wound closure rate or inflammatory response to wounding influenced tumour incidence, full-thickness skin wounds of different sizes (2, 4, 5, 6 and 8 mm²) were made on back skin of WT and InvEE (Inv) mice, and papilloma formation at the wound site was monitored. Wounds in InvEE and WT littermates healed at the same rate but only InvEE mice developed tumours (Supplementary Fig. 1a,b). Although onset of tumour formation was independent of wound size, there was a linear correlation between wound size and tumour incidence ($R^2 = 0.91381$; Fig. 1a,b). Wound size and total immune cell infiltrate (CD45⁺ cells) were correlated in both WT and InvEE skin, but CD45⁺ cells were significantly more abundant in InvEE skin both before wounding and at the time of wound closure (Fig. 1c and Supplementary Fig. 1c,d). There were even more CD45⁺ cells in the tumour stroma than in newly closed 8 mm² InvEE wounds (Fig. 1c). These results indicate that the degree of inflammation remaining once the acute response to injury has resolved correlates with the extent of the primary insult and subsequent tumour incidence.

As nuclear factor-κB (NF-κB) is an important mediator of inflammation-associated cancer, we analysed expression of NF-κB target genes in InvEE and control epithelium. All 16 of the genes examined were significantly upregulated in InvEE epidermis relative to WT epidermis ($P < 0.0001$ for each individual gene product; Fig. 1d). The effects were systemic, as levels of thymic stromal lymphopoietin (TSLP), tumour-necrosis factor (TNF)-α and interleukin (IL)-6 were elevated in serum of tumour-free InvEE mice and increased further in tumour-bearing animals (Fig. 1e–g).

**Tumour formation requires haematopoietic TNFR signalling.**

TNF-α is well known for its context-dependent pro- and anti-tumorigenic roles and downstream TNF-α signalling is mediated by TNFR-1 and TNFR-2. Mice deficient in both receptors are resistant to skin cancer induced by chemical carcinogens. To examine whether wound-induced tumorigenesis was dependent on TNFR signalling specifically in leukocytes, sub-lethally irradiated InvEE mice were reconstituted with TNFR⁻⁻⁻⁻ mice bone marrow (BM) and subsequently wounded. Successful engraftment was verified by Y chromosome-fluorescence in situ hybridization (Y-FISH) in spleens of reconstituted mice as previously described (Supplementary Fig. 2a).

TNF⁻⁻⁻⁻ chimeric mice were highly resistant to wound-induced tumour formation (Fig. 2a). Only 8.3% of TNFR⁻⁻⁻⁻ chimeric mice developed papillomas, compared with 50% of control chimeras, and time of tumour onset was delayed in TNF⁻⁻⁻⁻ chimeric mice (Fig. 2a). Although wounds closed more rapidly in TNF⁻⁻⁻⁻ chimeras (Supplementary Fig. 2b), in agreement with observations on TNFR-1⁻⁻⁻⁻ mice, the epidermis remained thickened and hyperproliferative, consistent with the ability of MEK1 to stimulate keratinocyte proliferation in the absence of other cell types (Fig. 2b). Serum levels of TNF-α were markedly reduced in tumour-free but not tumour-bearing TNF⁻⁻⁻⁻ chimeras (Supplementary Fig. 2c), consistent with MEK1 activation in epidermal tumour cells driving NF-κB activation.

The tumour-protective effect of TNFR ablation in radiosensitive leukocytes correlated with changes in the skin immune cell infiltrate. CD4⁺ T cells were markedly reduced in wounds and papillomas of TNF⁻⁻⁻⁻ BM chimeras (Fig. 2c,d). When irradiated InvEE mice were reconstituted with BM from mice expressing enhanced green fluorescent protein under the control of the β-actin cytomegalovirus (CMV) promoter and subsequently wounded, both the wounds and wound-induced tumours were heavily infiltrated with F4/80⁺ macrophages (Supplementary Fig. 2d). Macrophage (F4/80⁺ CD11b⁺) and mast cell numbers were similar in healed wounds of TNF⁻⁻⁻⁻ and control chimeras but significantly reduced in tumour stroma of TNF⁻⁻⁻⁻ chimeras (Fig. 2e–g).

Epidermal γδ T cells infiltrated wounds of both TNF⁻⁻⁻⁻ and control chimeras to the same extent. They were never present within the tumour epithelium, but did accumulate in adjacent epidermis (Supplementary Fig. 2e), suggesting that the previously observed reduction in tumours on γδ T-cell ablation is an indirect effect of reduced macrophage recruitment. TNF ablation in the BM did not affect numbers of dendritic cells (CD207⁺ CD11c⁻), NK or NKT cells infiltrating wounds or tumours (Supplementary Fig. 2f–h). B cells (CD19⁺) were not detectable in unwounded skin or healed wound beds and there was no difference in the stromal B-cell content of TNF⁻⁻⁻⁻ and control chimeric tumours (Supplementary Fig. 2i).

We conclude that TNFR ablation in leukocytes protected mice from developing tumours. It also led to a selective reduction in CD4⁺ T cells in wounded skin and a reduction in several immune cell subsets in tumour stroma.

**MyD88 and TLR-5 signalling mediate tumour formation.**

MyD88 (Myeloid Differentiation primary response gene 88) is a
master regulator of innate signalling events as it is the key adaptor for most TLRs, IL-1R1 and IL-1R8. Loss of MyD88 prevents tumour formation in various tissues. Given that MyD88 controls TNF-α production, we analysed the effect of reconstituting InvEE mice with MyD88−/− radiosensitive leukocytes. BM chimeras lacking MyD88 in the haematopoietic compartment exhibited a striking protection against wound-induced tumour formation (Fig. 3a).

Although InvEE keratinocytes express elevated levels of IL-1α and administration of the IL-1 receptor antagonist Kinere decreases tumour formation, no differences in wound-induced tumour formation were observed between IL-1R1−/− BM and control chimeras (Fig. 3b). We therefore examined the effects of deleting TLRs. Replacement of the radiosensitive haematopoietic compartment with TLR-2/4−/− or TLR-9−/− cells did not affect tumour formation (Fig. 3c), in contrast to the role of TLR-4 on haematopoietic and non-haematopoietic cells in chemically induced skin carcinogenesis. InvEE mice reconstituted with TLR-7/8−/− BM exhibited accelerated wound closure (Supplementary Fig. 3a) but no difference in tumour incidence was observed (Fig. 3d). TLR-3 and TLR-4 can signal via TIR-domain-containing adapter-inducing interferon-β (TRIF), instead of MyD88. However, reconstitution with TRIF−/− BM cells had no effect on papilloma formation (Fig. 3e).

Ablation of TLR-5 in radiosensitive leukocytes markedly reduced the number of tumours that developed on wounding (Fig. 3d). Wound closure rates were similar in TLR-5−/− and control BM chimeras, suggesting that the dynamics of wound closure does not affect wound-induced tumour formation (Fig. 3f).

These findings reveal the significance of an innate MyD88–TLR-5-sensing axis specifically in BM-derived leukocytes that drives wound-induced tumour initiation.

Bacterial products mediate tumour initiation in InvEE mice. As flagellin (Fla), the sole known TLR-5 ligand, is the main protein constituent of bacterial flagella, we analysed whether...
lowering the microbial content of the skin would affect wound-induced tumour incidence. When mice were treated with the broad-spectrum antibiotic enrofloxacin (enr), either by administration in drinking water or topical application, the skin bacterial load was decreased (Supplementary Fig. 3c, d) and wound-induced tumour formation was greatly reduced (Fig. 4a,c). Tumour size in antibiotic-treated mice was greatly diminished compared with control chimeras treated with flagellin (Fig. 4g).

Flagellated bacterial strains are commensals on murine skin. When we labelled unwounded InvEE skin with an antibody to the flagellated *Escherichia coli* (*E. coli*) strain K12, we observed strong immunoreactivity in hair follicles, sebaceous glands and cornified skin layers (Fig. 4h), in agreement with a previous report. As expected, *E. coli* labelling was markedly reduced in antibiotic-treated skin. All epidermal layers stained positive for K12 *E. coli* in InvEE healed wound beds and papillomas (Fig. 4h).

Taken together, these data demonstrate that exposure to bacterial flagellin sensed by TLR-5 on radiosensitive leukocytes promotes tumour formation in InvEE mice.

### Role of TLR-5 signalling in carcinogen-induced tumours

To validate our observations in a second experimental setting, we induced tumours in WT mice via the classic two-stage DMBA/TPA (7,12-dimethylbenz(a)anthracene and 12-O-tetradecanoylphorbol-13-acetate) chemical carcinogenesis protocol in which DMBA induces H-Ras mutations and TPA causes chronic inflammation, promoting tumour development. Irradiated WT mice were reconstituted with WT (control) or TLR-5−/− BM and topically treated with DMBA. Mice subsequently received repeated applications of TPA with or without prior wounding (Fig. 5a). Wound closure was accelerated in TLR-5−/− BM chimeras treated with TPA (Supplementary Fig. 4a).
Mice treated with DMBA, but not TPA, and subsequently wounded, did not develop tumours (data not shown). Control chimeras that were wounded before TPA treatment developed tumours after 2 weeks of promotion, which is significantly faster than mice treated with TPA only (Fig. 5). There was a substantial delay in the development of DMBA/TPA-induced tumours in wounded TLR-5/Inv; C0/C0 BM chimeras relative to wounded WT (Fig. 5b). The tumour-protective effect of TLR-5 was also apparent in non-wounded DMBA/TPA-treated mice, albeit less marked (Fig. 5c). TLR-5/−/− BM reconstitution did not reduce the final number of papillomas that formed (Supplementary Fig. 4b,c) but decreased tumour size considerably (Fig. 5d,e).

We conclude that TLR-5-mediated signalling is involved in tumour initiation in two different skin cancer models.

**Upregulation of HMGB1 in wound-induced tumours.** To investigate the relevance of mouse wound-induced tumour formation to human skin cancer, we analysed SCCs from RDEB patients. RDEB is a rare skin blistering condition in which repetitive cycles of wounding and repair predispose the skin to the development of SCCs.5

HMGB1 is a nuclear danger-associated molecular pattern that is passively released from necrotic cells and actively secreted by inflammatory cells.28,29 HMGB1 is upregulated in RDEB patients30,31 and HMGB1 serum levels correlate with RDEB disease severity.30 HMGB1 is also upregulated in a mouse RDEB model and mediates recruitment of BM-derived cells in injured tissue.31 Furthermore, HMGB1 is induced in epithelial cells upon exposure to flagellin.32 We therefore investigated HMGB1 as a candidate biomarker linking human and mouse wound-associated skin cancer.

In lesional skin from RDEB patients, HMGB1 was highly upregulated compared with normal human skin and there was strong immunoreactivity for HMGB1 in epidermis and dermis (Fig. 6a; n = 6 patients per group). There was an even greater increase in HMGB1 immunoreactivity in RDEB SCCs (Fig. 6a; n = 6 patients). Although HMGB1 was mainly nuclear in normal human skin, we observed cytoplasmic HMGB1 in lesional skin and SCCs from RDEB patients (Fig. 6a), which is indicative of HMGB1 secretion in these inflammatory conditions.29 Consistent with these findings, HMGB1 was elevated in unwounded InvEE skin relative to WT (Fig. 6b,c) and further increased on wounding in wound-induced papillomas (n = 8; Fig. 6b,c). HMGB1 expression was significantly downregulated in skin of unwounded TLR-5/−/−/Inv relative to Inv/Inv BM chimeras and the absence of TLR-5 prevented HMGB1 upregulation on wounding (Fig. 6c). The reduced immunolabelling in skin correlated with a reduction in serum HMGB1 levels in wounded mice (Fig. 6d).

We conclude that HMGB1 is upregulated in wound-associated mouse and human skin tumours and that HMGB1 levels are regulated by leukocytic TLR-5 signalling.
bacteria in wound-induced skin cancer. We demonstrate that TLR-5 ablation in BM-derived leukocytes reduced tumour incidence in mice and injection of the TLR-5 ligand flagellin induced papillomas in a TLR-5-dependent manner. Our analysis of RDEB tumours points to the relevance of our findings to

**Discussion**

Host–microbe interactions are crucial for tissue homeostasis and evidence showing cancer-promoting effects of the commensal microbiome in various organs is accumulating. Our studies establish, for the first time, a role for innate sensing of flagellated bacteria in wound-induced skin cancer. We demonstrate that TLR-5 ablation in BM-derived leukocytes reduced tumour incidence in mice and injection of the TLR-5 ligand flagellin induced papillomas in a TLR-5-dependent manner. Our analysis of RDEB tumours points to the relevance of our findings to
human skin cancer. HMGB1, which is induced in epithelial cells in response to flagellin\textsuperscript{32}, was strongly upregulated in wound-associated tumours in mice and RDEB patients. Ablation of TLR-5 in BM cells led to significantly reduced murine HMGB1 serum levels.

The skin microbiome composition is different across body surfaces\textsuperscript{36} and directs local immune responses\textsuperscript{26,37}. The human microbiome project reference genome database reveals that intact skin contains flagellated bacterial species (based on the presence of \textit{FliC} and \textit{FliA} genes; http://www.hmpdacc.org/catalog\textsuperscript{38}). Consistent with this, a flagellated \textit{E. coli} strain is present on InvEE skin\textsuperscript{26}. Although most bacteria are kept at bay by the skin barrier, in a wound situation the moist and metabolite-rich environment can promote growth of opportunistic bacteria, including energetically expensive flagellated species\textsuperscript{39}. Profiling the microbial configurations in chronic diabetic wounds and leg ulcers has indicated an increased representation of \textit{E. coli}, \textit{Pseudomonas aeruginosa}, \textit{Shigella} and other species comprising flagellated strains, whereas \textit{Staphylococci}, a genus that does not comprise flagellates, are negatively correlated with ulcer duration\textsuperscript{40,41}. Consistent with the observations in human skin, topical application of a broad-spectrum antibiotic was tumour protective in InvEE mice, whereas an antibiotic that targets \textit{Staphylococci} was not. These observations lead us to propose that a key, and previously unrecognized, driver of wound-induced skin cancer is an increased exposure of leukocytes to flagellated bacteria.

Both of the mouse models in which we demonstrated a role for leukocytic TLR-5 sensing in wound-induced skin cancer are

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\caption{TLR-5 signalling in leukocytes promotes DMBA/TPA wound-induced tumorigenesis. (a) Schematic of DMBA/TPA wound-induced tumorigenesis protocol. (b,c) Incidence of papilloma formation in WT mice reconstituted with TLR-5\textsuperscript{−/−} (WT/TLR5\textsuperscript{−/−}) or control (WT/WT) BM and treated with DMBA and TPA with (b) or without (c) wounding. (b) \(n = 10\) WT/WT mice; \(n = 9\) TLR5\textsuperscript{−/−}/WT mice; ****\(P<0.0001\); unpaired \(t\)-test. (c) \(n = 10\) WT/WT mice; \(n = 9\) TLR5\textsuperscript{−/−}/WT chimeras; **0.01 < \(P < 0.001\); unpaired \(t\)-test. (d) Back skin of representative wounded WT/WT and WT/TLR5\textsuperscript{−/−} mice, 4, 14 and 18 weeks after start of TPA treatment. (e) Average total number of tumours and tumour size measured weekly after first TPA treatment. \(n > 8\) mice per condition.}
\end{figure}
charakterized by chronic inflammation. In InvEE mice, the inflammation results from epidermal expression of constitutively active MEK1 and in WT mice inflammation is induced by TPA treatment. TLR-5 ablation in BM does not abolish the inflammatory infiltrate in InvEE skin or TPA-treated WT skin. Our studies indicate that in the context of chronic inflammation, TLR-5 signalling in leukocytes can tip the balance between normal wound repair and tumour formation. The tumour-inhibitory effect of ablating TNFR or MyD88 in radiosensitive leukocytes in InvEE mice was greater than that of TLR-5 ablation (Figs 2a and 3a,d; 0.01 < P < 0.05; one-way analysis of variance), suggesting that additional components of the immune system that converge on NF-kB may contribute (Fig. 1d).

Our findings raise the possibility that the incidence of SCCs in non-healing ulcers and skin from RDEB patients could be reduced by systemic antibiotics treatment and the use of flagellin-specific targeting strategies in wound-induced malignancies might present an interesting clinical avenue.

Methods

Mice. InvEE mice were maintained on an F1 genetic background (CBA × C57Bl/6) and kept heterogenous for the MEK1 transgene. Transgene negative littermates were used as WT controls and in DMBA/TPA experiments. All donor mice in the BM transplantation experiments were on a C57Bl/6 genetic background. TLR-5−/− and TNFR-1−/−/− mice were purchased from Jackson Laboratories. TLR-2/− and 4/− mice were obtained from Simon Clare, IL-1R1−/− mice from Nancy Rothwell, TRIF−/− mice from Frederic Geissmann, TLR-7/−/− mice from Lena Alexapoulou, MyD88−/− mice from Caetano Reis e Sousa and TLR-9−/− mice from Kinya Otsu. Animal procedures were subject to local ethical approval and performed under a UK Government Home Office Licence. Sample sizes were determined on the basis of prior power calculations. No mice died as a direct result of wounding or tumour formation. Mice that died as a result of myeloablation were excluded from analysis.

Wounding and BM reconstitution. Full-thickness wounds were made on the back skin by using 2, 4, 5, 6 or 8 mm² punch biopsy needles (Stiefel Instruments) under anaesthesia and general anaesthesia in 8- to 16-week-old age- and sex-matched InvEE and control littermates. Statistical power was calculated using the resource equation and animals were randomly assigned to treatment groups. No animals were excluded from any experiment. Tumour formation and size were measured by two independent researchers, who were blinded to group allocations.

In BM transplantation experiments, 8- to 16-week-old female recipient mice were treated with acidified water at least 10 days before irradiation. Allogenic BM transplants were performed 24 h after myeloablative total body irradiation (two times 5 Gy, separated by 3 h) of InvEE mice. Donor BM was isolated from the tibia and femur of male mice. BM reconstitution was performed by intravenous injection of 5 × 10⁶ BM cells in 200 μl of PBS 24 h after irradiation. Chimerism was confirmed using Y-chromosome in situ hybridization (Cytocell, probe AMPOYR) on spleens of reconstituted mice. Mice were wounded 4 to 6 weeks post BM reconstitution and papilloma formation was monitored for 50 to 60 days post wounding. Each InvEE BM reconstitution experiment was repeated two to three times.

Treatment with antibiotics and flagellin. Mice were treated with the broad-spectrum antibiotic enrofloxacin by oral administration (5 ml Baytril per liter drinking water) starting 8 days before wounding and continuing until the end of the experiment. For topical applications, shaved back skin of mice was treated daily with 200 μl of vehicle (acetone) or antibiotics in acetone starting 8 days before wounding. Antibiotics were used 0.25% enrofloxacin or 0.2 mg ml⁻¹ methicillin. Skin swabs were taken on day 10 post wounding and plated on lysogeny broth (LB) and blood agar plates to verify bacterial depletion. 16S rRNA quantification was performed on skin biopsies taken at the time of wounding. 1 or 4 μg flagellin (high-purity flagellin, EnzoLifeSciences) was injected once intradermally in back skin in 100 μl PBS; control mice were injected with 100 μl PBS. Flagellin was applied once topically to 8 mm² full-thickness skin wounds at time of wounding.

Quantitative PCR. Epidermis was separated from the underlying dermis by scraping skin after incubation at 60 °C for 7 s in RNase-free water. Isolated epidermis was homogenized in lysis buffer using bead beating. RNA extraction was performed according to the manufacturer’s instructions using a QIAGEN RNeasy Kit (Qiagen). Complementary DNA was generated using reverse transcriptase III (Invitrogen). Quantitative real-time PCR reactions were set up using gene-specific primer sets (see Supplementary Table 1) and reactions were performed on a 7900HT real-time PCR machine (Applied Biosystems) on biological triplicates.

Microbiota quantification. Bacterial load was quantified in skin biopsy samples (8 mm²) and in faeces collected from mice 8 days after antibiotic treatment. Samples were digested with Ready-Lyse Lysozyme Solution (Epiphenlab Bio- technologies) in lysate buffer (20 mM Tris, pH 8.0, 2 mM EDTA, 1.2% Triton X-100, DNA-free water). Samples were homogenized using 5 mm Stainless Steel Beads (Qiagen) in a Precellys homogenizer twice at 6,000 r.p.m. for 50 s. DNA was...
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