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LETTERS

IL-23 promotes tumour incidence and growth

John L. Langowski[1]*, Xueqing Zhang[1]*, Lingling Wu[1], Jeanine D. Mattson[1], Taiying Chen[1], Kathy Smith[1], Beth Basham[1], Terrill McClanahan[1], Robert A. Kastelein[1] & Martin Oft[1]

Chronic inflammation has long been associated with increased incidence of malignancy and similarities in the regulatory mechanisms have been suggested for more than a century[1]. Infiltration of innate immune cells, elevated activities of matrix metalloproteases and increased angiogenesis and vasculature density are a few examples of the similarities between chronic and tumour-associated inflammation[2]. Conversely, the elimination of early malignant lesions by immune surveillance, which relies on the cytotoxic activity of tumour-infiltrating T cells or intra-epithelial lymphocytes, is thought to be rate-limiting for the risk to develop cancer[3]. Here we show a molecular connection between the rise in tumour-associated inflammation and a lack of tumour immune surveillance. Expression of the heterodimeric cytokine interleukin (IL)-23, but not of its close relative IL-12, is increased in human tumours. Expression of these cytokines antagonistically regulates local inflammatory responses in the tumour microenvironment and infiltration of intra-epithelial lymphocytes. Whereas IL-12 promotes infiltration of cytotoxic T cells, IL-23 promotes inflammatory responses such as upregulation of the matrix metalloprotease MMP9, and increases angiogenesis but reduces CD8 T-cell infiltration. Genetic deletion or antibody-mediated elimination of IL-23 leads to increased infiltration of cytotoxic T cells into the transformed tissue, rendering a protective effect against chemically induced carcinogenesis. Finally, transplanted tumours are growth-restricted in hosts depleted for IL-23 or in IL-23-receptor-deficient mice. Although many strategies for immune therapy of cancer attempt to stimulate an immune response against solid tumours, infiltration of effector cells into the tumour tissue often appears to be a critical hurdle[4–7]. We show that IL-23 is an important molecular link between tumour-promoting pro-inflammatory processes and the failure of the adaptive immune surveillance to infiltrate tumours.

IL-12 and IL-23 are members of a small family of pro-inflammatory heterodimeric cytokines[8]. Both cytokines share a common p40 subunit that is covalently linked either to a p35 subunit to form IL-12 or to a p19 subunit to form IL-23 (ref. 9). The receptor for IL-12 is comprised of an IL-12Rβ1 and IL-12Rβ2 subunit, whereas the receptor for IL-23 is comprised of the IL-12Rβ1 subunit and a novel component termed IL-23R[10]. Both cytokines are expressed predominantly by activated dendritic cells and phagocytic cells. Receptors for both cytokines are expressed on T cells, natural killer cells and natural killer T cells, but low levels of IL-23 receptor complexes are also found on monocytes, macrophages and dendritic-cell populations[10]. Despite these similarities, there is increasing evidence that IL-12 and IL-23 drive divergent immunological pathways. Whereas IL-12 leads to development of 'classical' interferon-γ-producing Th1 cells and enhances cytotoxic, antimicrobial and anti-tumour responses, IL-23 drives a pathway that leads to the generation of IL-17-producing $CD4^+$ T cells[11–13]. The induction of IL-23-derived processes leads to recruitment of a range of inflammatory cells as well as T_HIL-17 T cells, and has been shown to be crucial to the pathogenesis of a number of immune-mediated inflammatory diseases[14].

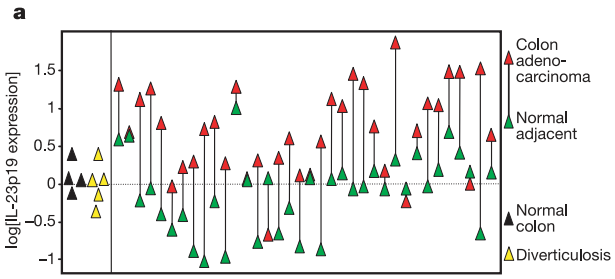
Given the combined evidence of a causal relationship between chronic inflammation and cancer as well as the pivotal role IL-23 plays in autoimmunity, we hypothesized that IL-23-mediated responses may be important in tumour promotion. To test this, we employed a comprehensive examination of messenger RNA expression within various human cancer panels. Surprisingly, IL-23p19 mRNA was found significantly upregulated in the overwhelming majority of carcinoma samples from various organ types when compared with their adjacent normal tissue (Fig. 1a, b). Significant mRNA upregulation was observed for both subunits of IL-23 (Fig. 1c), but not for the IL-12-specific subunit p35 (Supplementary Fig. S2). The expression of IL-17, a cytokine central to tumour-promoting angiogenesis[15], was also found to be significantly elevated in human tumours, consistent with activation of IL-23-induced processes (Supplementary Fig. S2 and data not shown). Additionally, human colorectal tumours present a high degree of correlation between increased IL-23p19 expression and increased macrophage and neutrophil infiltration activation[16] (Supplementary Fig. S3). Expression of IL-23p19 protein was localized within human tumour tissue but not in the surrounding stroma, and observed on infiltrating $CD11c^+$ dendritic cells as a dominant source of the cytokine (Supplementary Fig. S4a, b). Supporting this, tumour-infiltrating macrophages ($CD11b^+$) and dendritic cells ($CD11c^+$) isolated from mouse syngeneic tumour models expressed preferentially high amounts of IL-23p19 mRNA rather than IL-12p35 when compared to those isolated from the spleen (Supplementary Fig. S4c, d).

To examine more directly the role of IL-12 and IL-23 in epithelial tumorigenesis, we employed a strategy of genetic deletion, analysing the susceptibility of mice deficient in either IL-12 or IL-23 ($p35^{-/-}$ or $p19^{-/-}$ mice) or mice deficient in both cytokines ($p40^{-/-}$ mice) to tumour formation during chemical carcinogenesis[17]. Whereas $Il12p35^{-/-}$ mice showed earlier appearance and developed significantly increased numbers of papillomas compared to C57BL/6 control mice (Fig. 2a, b), mice deficient in IL-23p19 were resistant to tumour induction. Surprisingly, $p40^{-/-}$ mice, deficient in both cytokines, were resistant to tumour induction, emphasizing the importance of IL-23 for increased tumour incidence. Papillomas generated by this protocol persisted well over 100 days after cessation of TPA promotion (see Methods) and often progressed towards malignancy (Fig. 2b and data not shown); however, the low overall incidence of benign tumours in the absence of IL-23 prevents a statistically relevant comparison with WT mice in this regard.

To explore the role of IL-23 in tumour susceptibility, we examined the expression of inflammatory immune cell products in carcinogen-treated skin. IL-23 is known to modulate the homeostasis of

[1]Schering-Plough BioPharma (formerly DNAX Research, Inc.), 901 California Avenue, Palo Alto, California 94304, USA.
*These authors contributed equally to this work.

neutrophil infiltration into tissues by inducing expression of IL-17 and G-CSF[18]—two cytokines that are also linked to tumour growth[15,19]. We found IL-17 highly expressed in the hyperplastic skin of both wild-type C57BL/6 (WT) and *Il12p35*$^{-/-}$ mice, but barely detectable in *Il23p19*$^{-/-}$ and *Il12p40*$^{-/-}$ mice. Similarly, G-CSF levels were decreased in *Il23p19*$^{-/-}$ mice (Fig. 2c). The number of granulocytes (GR1^{+}) and macrophages (CD11b^{+}, F4/80^{+}) in the dermis of *Il23p19*$^{-/-}$ and *Il12p40*$^{-/-}$ mice was also decreased compared to the skin of C57BL/6 and *Il12p35*$^{-/-}$ mice (Fig. 2d, Supplementary Fig. S5a, b and data not shown). In murine cancer models, tumour development is dependent on MMP9 activity produced by inflammatory macrophages and mast cells[20]. Additionally, MMP9 and IL-17 have been identified as both markers and mediators of tumour angiogenesis[15,21]. The expression of activated MMP9 was elevated in carcinogen-treated skin of tumour-prone WT and *Il12p35*$^{-/-}$ animals, but dramatically lower in skins of *Il23p19*$^{-/-}$ and *Il12p40*$^{-/-}$ mice (Fig. 2e–h, Supplementary Fig. S5d, e). Angiogenic markers also followed this pattern, with significantly lower vessel density in IL-23p19-deficient animals (Fig. 2i–l, Supplementary Fig. S5c). Thus, the absence of IL-23 resulted in a significant reduction in numerous inflammatory factors and cell types essential for tumour promotion.



b

Cancer type	Number of paired (tumour and normal) samples	Fold increase in expression: Average	Number >5×	Number >10×	*P*
Colon	36	15.33	23	17	0.0001
Ovarian	32	9.45	12	4	0.0001
Head and neck	44	3.41	11	4	0.01
Lung	114	3.03	20	8	0.0001
Breast	78	2.86	16	6	0.0001
Stomach	64	2.13	9	3	0.001
Melanoma	89	1.47	5	0	0.0001

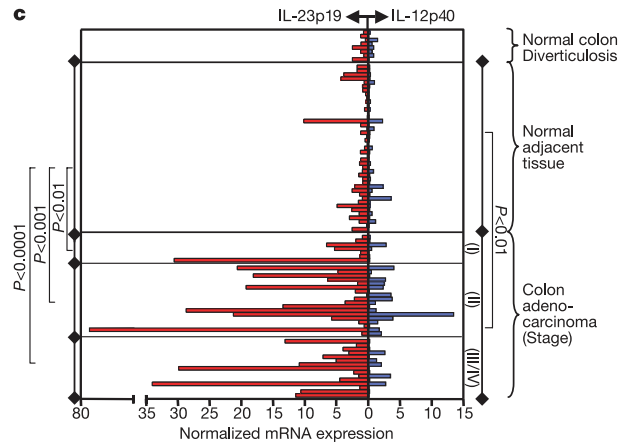


Figure 1 | Overexpression of IL-23 but not of IL-12 in human cancer. **a**, Quantitative mRNA expression of IL-23p19 in human colon tumour (red) compared to normal adjacent tissue of the same individual (green, connected by a line), or tissue from cancer-free individuals (black, yellow). **b**, Significant upregulation of IL-23p19 expression in various human cancers. The table shows number of individual paired cancerous and normal adjacent samples with average fold increase in expression, number of samples with five- and ten-fold increase, and *P*-value. **c**, Expression of both IL-23 subunits is increased in human cancer; each *y*-axis position signifies expression within an individual sample. Histological classification of samples as normal, normal adjacent and cancerous (with stage classification) is given.

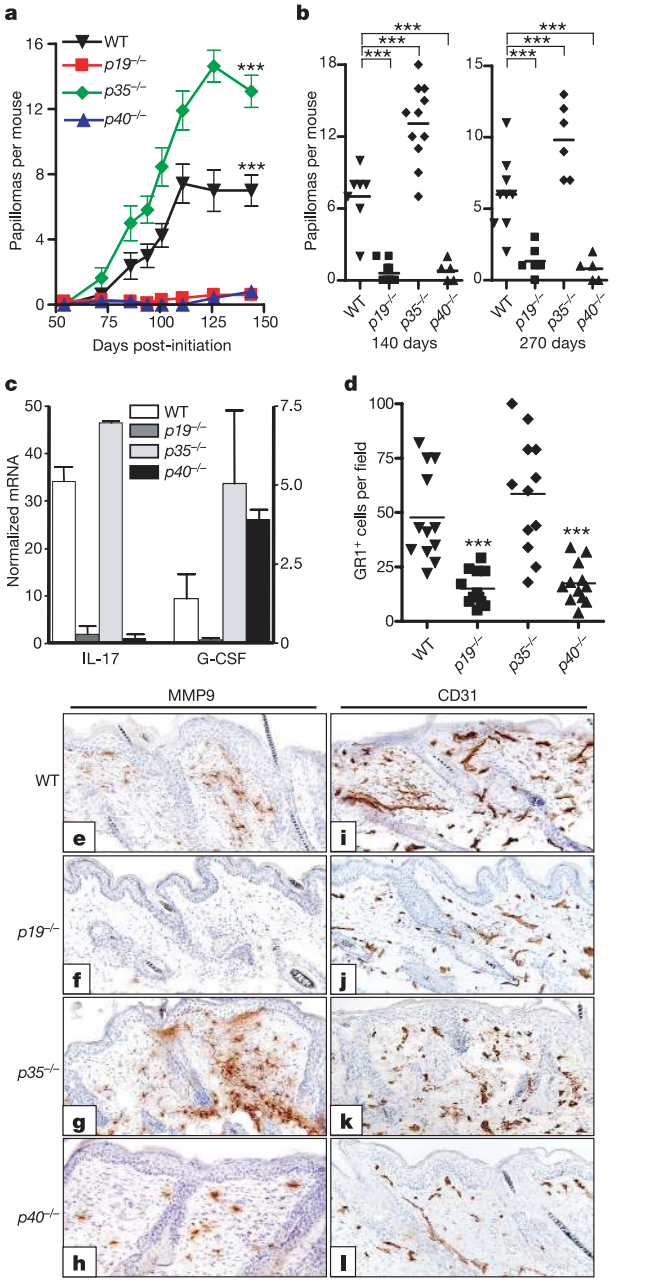


Figure 2 | Tumour resistance with reduced inflammation in IL-23-deficient animals. **a**, **b**, IL-23-deficient mice are resistant, whereas IL-12-deficient mice are more susceptible, to cutaneous chemical carcinogenesis. Papillomas persisted more than 100 days following cessation of treatment (**b**, right panel). **c**, Cutaneous IL-17 and G-CSF mRNA expression following 140 days of carcinogenesis. **d**, Increased granulocyte infiltration in the dermis of WT and *Il12p35*$^{-/-}$ mice. **e**–**l**, Cutaneous expression of activated MMP9 (**e**–**h**) and the endothelial marker CD31 (**i**–**l**) following 140 days of carcinogenesis. We note increased angiogenesis in C57BL/6 and *Il12p35*$^{-/-}$ mice. Data are mean ± s.d.; scatter plots show incidence with bars depicting the mean. The significance of results in the figures is represented by: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$; $n \geq 7$ (**a**, **b**), $n = 3$ (**c**, **d**) per group.

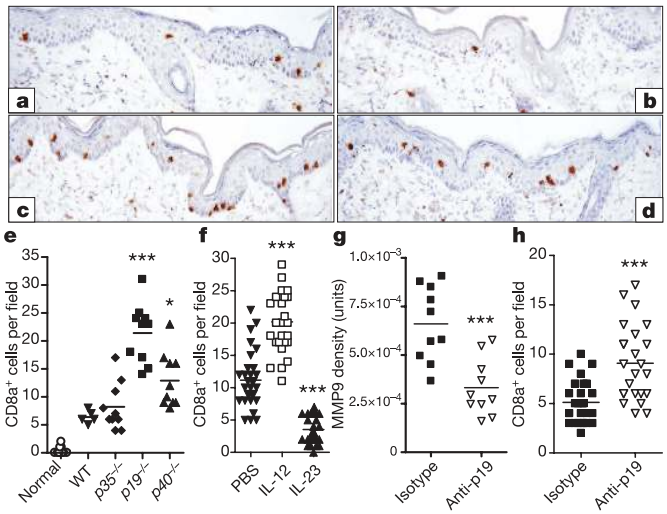


Figure 3 | IL-12 stimulates, but IL-23 decreases, immune surveillance by CD8 T cells. **a–e**, Increased immune surveillance by CD8 T cells in the epidermis of *Il23p19*$^{-/-}$ mice (**c**) and *Il12p40*$^{-/-}$ mice (**d**) treated with carcinogen for 140 days, compared to WT (**a**) and *p35*$^{-/-}$ (**b**). Quantification of infiltrating CD8 T cells for each genotype is shown in **e**. **f**, Alterations in epithelial CD8 infiltration following treatment with exogenous IL-23 or IL-12 compared to control-treated animals. **g**, **h**, Systemic treatment with anti-IL-23p19 antibody shows marked decrease in MMP9 expression (**g**), but increase in CD8$^+$ T cells (**h**), compared to control. Scatter plots show incidence or density with bars depicting the mean. Significance of results in figures is represented by: $^*P < 0.05$ and $^{***}P < 0.001$; $n = 3$ per group.

Because the incidence of macroscopic tumours can be viewed as the result of a disturbed balance between tumour-promoting factors and tumour-cell-eliminating mechanisms, our observations raised the question of whether endogenous IL-23 would have a direct role in immune surveillance against tumours. Immune surveillance and anti-tumour responses are vigorously promoted by IL-12, which induces proliferation and cytotoxic activity of natural killer cells and CD8$^+$ T cells[22]. Surprisingly, the carcinogen-induced hyperplastic skin of *Il23p19*$^{-/-}$ and *Il12p40*$^{-/-}$ mice were infiltrated with a dramatically increased number of cytotoxic CD8$^+$ T cells in both the dermis and the epidermis when compared to WT and *p35*$^{-/-}$ mice (Fig. 3a–e). The expression of markers associated with the activity of cytotoxic T cells such as Fas ligand, perforin and granzymes were decreased in *Il12p35*$^{-/-}$ animals but increased in *Il23p19*$^{-/-}$ mice (Supplementary Fig. S6 and data not shown). Although deficiency of IL-12 or IL-23 does not alter the development of CD8 cells (ref. 13 and data not shown), it may alter the response necessary for tissue infiltration. To test this, we changed the microenvironment of the carcinogen-treated skin of C57BL/6 mice by serial intradermal injection of IL-12 and IL-23. While administration of IL-12 led to increased immune surveillance, as shown by the influx of greater numbers of CD8$^+$ T cells in the epidermis, IL-23 injection caused a significant decrease of CD8 T cells in the hyperplastic skin (Fig. 3f, Supplementary Fig. S7a–c). Cytotoxic activity markers such as perforin, granzyme, Fas ligand and interferon-γ increased on IL-12 administration, whereas IL-23 greatly raised mRNA levels of IL-17 (Supplementary Fig. S7d, e). Systemic treatment with neutralizing antibodies specific to IL-23p19 led to a marked down-modulation of MMP9 expression and increased surveillance of CD8$^+$ T cells within the carcinogen-treated skin (Fig. 3g–h, Supplementary Fig. S8). Taken together, these results provide evidence that IL-23 negatively influences both the number and activity of cytotoxic T cells within transformed tissue.

To further investigate the effects of IL-12 and IL-23 on immune surveillance, IL-12- and IL-23-cytokine-deficient mice were challenged intradermally with PDV squamous carcinoma cells[17]. In accordance with the dominant role of IL-12 in tumour immunity, deficiency of IL-12p35 or IL-12p40 dramatically increased tumour incidence, indicating the diminished ability of the host to reject the

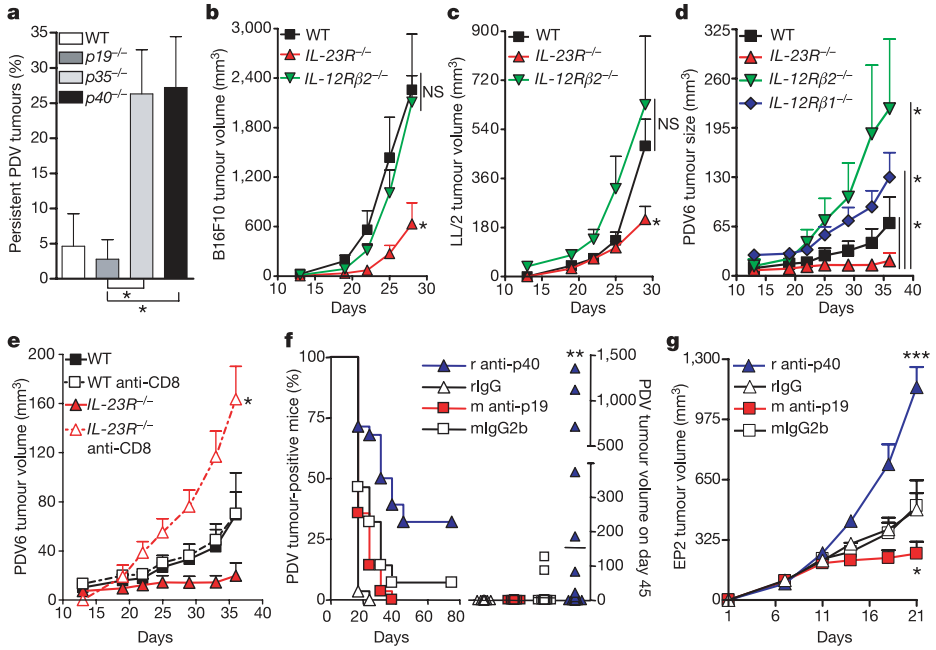


Figure 4 | Functional ablation of IL-23p19 signalling restricts tumour growth. **a**, Persistence of immune-sensitive PDV squamous carcinoma in IL-12-deficient but not WT or *p19*$^{-/-}$ mice. **b**, B16F10 melanoma and **c**, LL/2 lung carcinoma cells exhibit reduced growth in *Il23R*$^{-/-}$ mice. **d**, Tumour growth of immune-resistant PDV6 squamous carcinoma cells in IL-12- or IL-23-receptor-deficient mice. **e**, Growth restriction of PDV6 tumours is CD8-dependent only in *Il23R*$^{-/-}$ mice. **f**, Antibody ablation of IL-12p40 increases both tumour incidence and size compared to anti-IL-23p19 treatment. **g**, EP2 breast tumour growth is increased by ablation of IL-12p40 or reduced by anti-IL-23p19 treatment (***/* for days 18 and 21). Data are mean ± s.d.; scatter plots show incidence with bars depicting the mean. Significance of results in figures is represented by: $^*P < 0.05$ and $^{***}P < 0.001$; NS, not significant; $n \geq 5$ (**a–e**, **g**), $n \geq 14$ (**f**) mice per group with two tumours per mouse for all studies.

nascent carcinomas (Fig. 4a). IL-23p19-deficient mice, however, had a strong resistance to tumour challenge, similar to that of C57BL/6 control mice. Although previous reports suggest genetically engineered tumours overexpressing an artificially linked IL-23p19 and p40 construct are rejected more efficiently[23], our results show that at the very least IL-23 deficiency does not impair the ability of the immune system to eliminate nascent tumours.

To explore the respective importance of the host's immune response to IL-12 and IL-23 and to control tumour growth, we challenged mice incapable of responding to IL-23 (*Il23R*$^{-/-}$ mice) with subcutaneous transplantation of murine tumour cell lines. The growth of B16F10 melanoma and LL2 lung carcinoma was inhibited in *Il23R*$^{-/-}$ mice incapable of responding to IL-23, compared to either WT or mice incapable of responding to IL-12 (Fig. 4b, c and data not shown). We then used an immune-resistant subclone of the PDV squamous carcinoma (termed PDV6), to investigate the role of IL-12 and IL-23 signalling in the host on tumour growth and CD8 T-cell function. Growth of squamous carcinomas in *Il23R*$^{-/-}$ was reduced compared to that in WT mice, but increased in mice not able to respond to IL-12 (Fig. 4d). Although depletion of CD8 T cells in WT mice did not alter PDV6 tumour growth kinetics, it potentiated tumour growth in *Il23R*$^{-/-}$ mice (Fig. 4e). These results demonstrate that the absence of IL-23 signalling attenuates tumour growth and suggests an essential role for CD8 T cells in restricting tumour growth in *Il23R*$^{-/-}$ mice.

We next asked whether antibody ablation of IL-23 or combined ablation of IL-23 and IL-12 would have a different effect on the ability of the host immune system to eliminate early malignant tumour cells. Animals treated with anti-IL-23p19 showed a decreased risk of PDV tumour formation and faster elimination of injected tumour cells. However, animals treated with anti-IL-12p40, and thus depleted of both IL-12 and IL-23, had a significantly increased tumour incidence and developed larger, faster-growing tumours (Fig. 4f and data not shown). Finally, animals bearing tumours formed by a transformed mammary cell line were treated with IL-23 or IL-12 neutralizing antibodies. Mice treated with anti-IL-23p19 showed decreased tumour growth and increased tumour rejection. However, anti-IL-12p40 treatment led to a marked increase of tumour growth and an increase of metastasis formation (Fig. 4g and data not shown). Thus, although elimination of transformed cells is regulated by IL-12 (ref. 22), the contribution of the pro-inflammatory environment to tumour propagation can be seen in the result of functional IL-23 ablation.

The importance of inflammatory processes in tumour progression and initiation is exemplified by the tumour resistance of MMP9- or COX-2-deficient mice[20,24]. However, local expression of growth factors and cytokines in tumours may not only aid tissue remodelling and angiogenesis, but also protect the tumour from immune-mediated elimination[7]. Surprisingly, our work shows that IL-23 appears not only to induce innate inflammatory infiltration, but also to downregulate dramatically the ability of CD8 T cells to infiltrate tumours (Supplementary Fig. S1). Although the understanding of the biological role of IL-23 is still evolving, it is known that IL-23 production by dendritic cells and macrophages can be elicited by bacterial exposure and Toll-like receptor engagement. The resulting IL-17-dependent immune response constitutes a significant component for rapid neutralization of infectious organisms[9,25,26]. However, inappropriate expression of IL-23 also appears to coincide with numerous autoimmune disorders[14,27]. IL-23-induced inflammatory processes such as induction of angiogenesis or macrophage and neutrophil infiltration are valuable defence mechanisms against bacterial infections. However, this regulatory pathway appears to be not just inappropriate for eliminating tumours but instead could provide a protective, tumour-promoting environment for nascent malignancies, suggesting that anti-IL-23p19 therapy may prove efficacious for tumour treatment.

METHODS

Animal models. C57BL/6 mice and *Il12p35*$^{-/-}$ (*Il12a*^{tm1Jm}), *Il12p40*$^{-/-}$ (*Il12b*^{tm1Jm}), *Il12Rβ1*$^{-/-}$ (*Il12rb1*^{tm1Jm}), and *IL12Rβ2*$^{-/-}$ (*Il12rb2*^{tm1Jm}) mice on the C57BL/6 background were obtained from Jackson Laboratory. *Il23R*$^{-/-}$ (*Il23ra*^{tm1dnax}) were maintained on a C57BL/6 background; *Il23p19*$^{-/-}$ (*Il23a*^{tm1dnax}) were produced by DNAX[13] and made congenic on the C57BL/6 background (see Supplementary Methods). The two-step skin carcinogenesis model was adapted from ref. 28: mice were treated with 9,10-dimethyl-1,2-benzanthracene (DMBA; Sigma) in 200 μl acetone at 100 μg per mouse once at the age of 2–3 months, then treated twice-weekly with the tumour promoter 12-O-tetradecanoyl-phorbol acetate (TPA; Fisher) in 200 μl acetone, 30 μg per mouse for up to 1 year. Tumours observed arise as papillomas (keratoacanthomas) but statistically progressed towards carcinomas and metastasized via the lymph drainage. Papilloma counts were conducted routinely by visual examination. All experiments were repeated at least two times with similar significance values ($P = 0.0106$ and $P < 0.001$, respectively, $n \geq 7$ for each group in each experiment, with a total of $n \geq 21$ per genotype). Mice 8–10 weeks old were exposed to carcinogen for five months—they were either injected subcutaneously with murine IL-12, IL-23 or PBS at 20 ng per injection site or, for antibody ablation, systemically injected with 100 μg of anti-IL-23p19 antibody or isotype control. In both cases, skin was harvested 72 h later for immunohistochemistry or for TAQMAN gene expression analysis.

In tumour transplantation studies, transformed cell lines were mixed 1:1 with growth-factor-reduced Matrigel (Gibco) and injected subcutaneously or intradermally, typically in two separate sites per animal. Squamous carcinoma cells (PDV[29]), immune resistant PDV derivative (PDV6; DNAX), melanoma (B16F10; ATCC) and lung (LL2; ATCC) cell lines were injected into C57BL/6 mice, while breast (EP2; DNAX and 4T1; ATCC) and colon (CT26; ATCC) cancer cells were injected into BALB/c mice. For persistence of PDV transplants, tumour growth was followed for an observation period of 2–6 months. Anti-cytokine antibody injections (1 mg per mouse) were repeated weekly, and expected serum concentrations were confirmed after one week and at the end of the experiment. For anti-CD8 antibody ablation, 0.25 μg per mouse of antibody (DNAX) was injected subcutaneously once per week; ablation effectiveness was judged by flow cytometry of treated spleens at the experimental endpoint. Tumour volume was determined by electronic calliper measure: $(\text{half-length})^2 \times \text{width}$.

Antibodies, cytokines, immunohistochemistry, zymogram. Recombinant murine IL-12 was obtained from R&D, while rmIL-23 was produced at DNAX as previously described[9]. For the ablation of IL-12, monoclonal antibody C17.8 (ref. 30) was used. Mouse anti-murine IL-23p19 antibodies were developed at DNAX. Antibodies were injected subcutaneously in equal PBS volume at 1 mg per mouse per week. Expected serum concentrations of C17.8 and anti-IL-23p19 were confirmed by immunoassays (ELISAs) specific for each antibody. Mouse skin biopsies were harvested from macroscopically tumour-free areas of carcinogen-exposed skin, snap-frozen, fixed and subjected to immunohistochemistry and counterstained with haematoxylin. Antibodies used include CD3, CD8, CD11b, CD11c, CD31 (BD Biosciences), active MMP9, perforin and F4/80 (R&D). Density of staining was determined by analysing multiple 20× fields with ImagePro Plus software (MediaCybernetics). Gelatin zymogram gels (Invitrogen) were performed following the supplier's instructions.

Gene expression analysis. For TAQMAN analysis, total RNA was isolated by standard methodologies and reverse transcribed. Expression analysis for marker-specific mRNA was measured using real-time quantitative PCR (ABI 5700) with SYBR Green PCR Mastermix (Applied Biosystems). Ubiquitin levels were measured in a separate reaction and used to normalize the data by the $\Delta - \Delta C_t$ method, using the mean cycle threshold (C_t) value for ubiquitin and the gene of interest for each sample; the expression $1.8^{C_{t(\text{ubiquitin})} - C_{t(\text{gene of interest})}} \times 10^4$ was used to obtain the normalized values (see Supplementary Methods). For murine samples, punch biopsies were taken from at least three independent, macroscopically and histologically tumour-free areas for each condition.

Tumour-infiltrating immune cell isolation. Subcutaneous 4T1 and CT26 tumour masses were dissected and digested with 2 mg ml^{-1} collagenase and 0.5 mg ml^{-1} DNase (Sigma). Single-cell suspensions were created and live cells collected by centrifugation over a Histopaque (Sigma) gradient. Similar isolations from naive spleen were performed for comparison. Collected cells were positively selected for CD11b or CD11c by magnetic selection (Miltenyi Biotec) according to the manufacturer's specifications.

Statistical analyses. The following statistical analyses were used. For data in Fig. 1 and Supplementary Fig. S1, we used the Mann–Whitney test (non-paired Wilcox)—nonparametric, group comparison. In Figs 2, 3, 4a–c and Supplementary Figs S3 and S4, we used the Student's *t*-test. In Fig. 4a–c, e–g, we used the Kruskal–Wallis test followed by Dunn's multiple comparison test (non-parametric), or

analysis of variance (ANOVA) (parametric) followed by Tukey's multiple comparison test. In Fig. 4d, we used Wilcoxon matched pairs test. All tests (other than in Supplementary Fig. S2) were performed using Prism 4 (GraphPad Software). Linear regression analysis (Supplementary Fig. S2) was performed with Spotfire DecisionSite software.

Human tumour panels, immunohistochemistry. Paired human tumour and normal adjacent tissues were obtained from patients undergoing therapeutic or routine surgery, respectively (see Supplementary Methods). All sample diagnoses and staging were confirmed internally by a pathologist. Immunohistochemistry was performed on frozen sections with murine anti-human IL-23p19 (DNAX) with biotinylated horse anti-mouse IgG (Vector Laboratories).

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