IL-17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis

Seth B. Coffelt1, Kelly Kersten1*, Chris W. Doornebal1*, Jorieke Weiden1, Kim Vrijland1, Chee-Sing Hau1, Niels J. M. Verstegen1, Metamia Ciampricotti1, Lukas J. A. C. Hawinkels2,3, Jos Jonkers4 & Karin E. de Visser1

Metastatic disease remains the primary cause of death for patients with breast cancer. The different steps of the metastatic cascade rely on reciprocal interactions between cancer cells and their micro-environment. Within this local microenvironment and in distant organs, immune cells and their mediators are known to facilitate metastasis formation14. However, the precise contribution of tumour-induced systemic inflammation to metastasis and the mechanisms regulating systemic inflammation are poorly understood. Here we show that tumours maximize their chance of metastasizing by evoking a systemic inflammatory cascade in mouse models of spontaneous breast cancer metastasis. We mechanistically demonstrate that interleukin (IL)-1β elicits IL-17 expression from gamma delta (γδ) T cells, resulting in systemic, granulocyte colony-stimulating factor (G-CSF)-dependent expansion and polarization of neutrophils in mice bearing mammary tumours. Tumour-induced neutrophils acquire the ability to suppress cytotoxic T lymphocytes carrying the CD8 antigen, which limit the establishment of metastases. Neutralization of IL-17 or G-CSF and absence of γδ T cells prevents neutrophil accumulation and downregulates the T-cell-suppressive phenotype of neutrophils. Moreover, the absence of γδ T cells or neutrophils profoundly reduces pulmonary and lymph node metastasis by out-influencing primary tumour progression. Our data indicate that this novel cancer-cell-initiated domino effect within the immune system—the γδ T cell/IL-17/neutrophil axis—represents a new strategy to inhibit metastatic disease.

In patients with breast cancer, increased neutrophil abundance predicts worsened metastasis-specific survival1,4. Currently, the role of neutrophils in metastasis is controversial, since both pro- and anti-metastatic functions have been described15–17. We found a profound systemic expansion of neutrophils in mammary tumour-bearing K14cre; Cdh1F/F;Trp53F/F (KEP) mice15, compared with wild-type (WT) littermates (Extended Data Fig. 1a, b). Neutrophils, defined as CD45+ CD11b+ Ly6G+ Ly6C+ F4/80− cells, accumulated throughout every organ examined (Extended Data Fig. 1c, d). We also investigated our recently described KEP-based model of spontaneous breast cancer metastasis15 (Fig. 1a), where systemic neutrophil expansion was observed as well (Fig. 1b and Extended Data Fig. 1e, f). Neutrophil expansion was tumour-induced, because surgical removal of the primary tumour resulted in their immediate reduction (Extended Data Fig. 1g).

To determine the functional significance of neutrophils in metastasis, neutrophils were depleted using anti-Ly6G antibodies (Extended Data Fig. 2a–c). Treatment was initiated when tumours were palpable and continued until mice developed overt metastatic disease. Neutrophil depletion did not influence tumour growth (Extended Data Fig. 2d), tumour histopathology (Extended Data Fig. 2e) or microvesSEL density (Extended Data Fig. 2e, f). In contrast, neutrophil depletion resulted in significant reduction in both pulmonary and lymph node metastasis (Fig. 1c). These data indicate that neutrophils assist the spread of cancer cells to multiple locations.

Next, we evaluated the role of neutrophils in different phases of the metastatic cascade. Neutrophils were depleted during primary tumour growth (early phase) or after removal of the primary tumour (late phase) (Fig. 1a). Interestingly, neutrophil depletion decreased multi-organ metastasis in the early phase, but not the late phase (Fig. 1d). Metastatic nodule size was not affected (Extended Data Fig. 2g), suggesting that neutrophils facilitate multi-organ metastasis during the early steps of the metastatic cascade.

To understand the mechanism by which neutrophils facilitate metastasis, their phenotype was investigated. Previous reports identified the haematopoietic stem cell marker cKIT on pro-metastatic myeloid cells15,16–18, and CD11b+ VEGFR1+ cells have been implicated in the pre-metastatic niche19–21. A greater proportion of neutrophils from tumour-bearing KEP mice expressed cKIT, while both neutrophils and monocytes from WT and KEP mice expressed VEGFR1 (Extended Data Fig. 3a, b). In the metastasis model, cKIT+ neutrophils also expanded systemically, as tumours grew larger (Extended Data Fig. 3c) and reduced to baseline levels after tumour resection (Extended Data Fig. 3d). Nuclear morphological analysis revealed characteristics of immature cells22, including banded, circular and non-segmented nuclei, whereas most WT neutrophils appeared hyper-segmented (Extended Data Fig. 3e), suggesting that KEP mammary tumours promote the release of immature neutrophils into circulation.

Next-generation RNA sequencing (RNA-seq) was performed on circulating neutrophils from WT and tumour-bearing KEP mice, revealing 100 differentially expressed genes (Extended Data Fig. 4a and Extended Data Table 1). Several genes upregulated in neutrophils from KEP mice, including Prok2 (also known as Bv8), S100a8 and S100a9 (Fig. 2a), have previously been linked to metastasis16–18. Nos2, the gene encoding inducible nitric oxide synthase (iNOS), was the most strongly upregulated gene, by more than 150-fold (Fig. 2a). Because iNOS suppresses T cells16–18, we hypothesized that neutrophils promote metastasis via immunosuppression. Indeed, neutrophils from KEP mice inhibited the CD3/CD28-induced proliferation of naïve splenic CD8+ T cells ex vivo compared with WT neutrophils, and an iNOS inhibitor reversed this effect (Fig. 2b and Extended Data Fig. 4b). In lungs of control and neutrophil-depleted tumour-bearing mice, the proportions of CD8+ T cells did not differ (Extended Data Fig. 4c). However, the effector phenotype of CD8+ T cells was markedly enhanced upon neutrophil depletion, as evidenced by a significantly greater proportion of CD62L− CD44+ and interferon-γ+ (IFN-γ+) cells (Fig. 2c and Extended Data Fig. 4d, e). To establish further a mechanistic link between neutrophils and CD8+ T cell activity, we depleted both cell populations in the metastasis model. Combined depletion of neutrophils and CD8+ T cells reversed the metastasis phenotype of neutrophil depletion alone.

1Division of Immunology, Netherlands Cancer Institute, Plesmanlaan 121, Amsterdam, 1066 CX, The Netherlands. 2Department of Molecular Cell Biology, Leiden University Medical Center, Albinusdreef 2, Leiden, 2300 RC, The Netherlands. 3Centre for Biomedical Genetics, Leiden University Medical Center, Albinusdreef 2, Leiden, 2300 RC, The Netherlands. 4Division of Molecular Pathology, Netherlands Cancer Institute, Plesmanlaan 121, Amsterdam, 1066 CX, The Netherlands. *These authors contributed equally to this work.

©2015 Macmillan Publishers Limited. All rights reserved
**Figure 1 | Neutrophils promote breast cancer metastasis.** a, Spontaneous metastasis model. Tumour fragments from KEP mice are orthotopically transplanted into WT female FVB/N recipient mice (designated by 1), allowed to proliferate (2), then surgically resected (3). Metastases develop in 100% of recipient mice. Antibody-mediated depletion experiments were performed in three ways: from palpable tumours to metastasis-related death (continuous treatment), during primary tumour growth (early phase) or after surgery until metastasis-related death (late phase). b, Neutrophil proportions in lungs at the indicated tumour size (n = 6, 5, 6 and 8 mice for 0, 9, 25 and 100 mm², respectively; Kruskal–Wallis test followed by post-hoc Dunn’s test). c, Images of cytoketerin 8-stained lung sections, quantification of lung metastases and incidence of metastasis in lymph nodes. Neutrophils were depleted continuously until metastasis-related death in c (n = 11 mice per group; Mann–Whitney U-test and Fisher’s exact test) or depleted during the early or late phases in d (n = 9 control, 11 early phase, 14 late phase; Kruskal–Wallis test followed by post-hoc Dunn’s test and Fisher’s exact test). Data in d are representative of two independent experiments. All data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001. Scale bars, 6 mm.

(Fig. 2d), without affecting primary tumour growth (Extended Data Fig. 4f). Depletion of CD8⁺ T cells alone did not alter tumour growth or multi-organ metastasis (data not shown). These data suggest that neutrophils facilitate cancer cell spread by suppressing CD8⁺ T cells. As such, neutrophils in the KEP model can be categorized as a subpopulation of myeloid-derived suppressor cells.

We then asked how mammary tumours induce systemic neutrophil expansion. Cytokine profile comparison of WT mammary glands and KEP mammary tumours showed that granulocyte–macrophage colony-stimulating factor (GM-CSF) and G-CSF levels—two key regulators of neutrophil biology—were not significantly increased in KEP tumours (Extended Data Fig. 5a, b). However, expression of IL-1β, IL-6 and IL-12p40, a subunit of IL-23, was increased (Extended Data Fig. 5a, b). These cytokines are known to stimulate IL-17 from lymphocytes.

In inflammatory diseases such as psoriasis, lymphocyte-derived IL-17 regulates neutrophil expansion via systemic induction of G-CSF. We hypothesized that the same inflammatory cascade is important in breast cancer metastasis. Indeed, serum levels of IL-17A and G-CSF were higher in tumour-bearing KEP mice than in WT mice (Fig. 3a). Neutralization of IL-17A in tumour-bearing KEP mice decreased G-CSF serum levels, while G-CSF blockade did not affect IL-17A levels (Fig. 3a), indicating that IL-17 is upstream of G-CSF. Inhibition of either cytokine reduced circulating neutrophils, lowered cKIT⁺ neutrophil proportions (Fig. 3b) and reversed neutrophil phenotype (Fig. 3c). Injection of recombinant G-CSF to anti-IL-17-treated tumour-bearing KEP mice overcame the effects of anti-IL-17 treatment (Extended Data Fig. 5c–e). Additionally, treatment of WT mice with recombinant G-CSF resulted in neutrophil expansion, increased presence of cKIT⁺ neutrophil proportions and changed neutrophil phenotype (Extended Data Fig. 5c–e). These data demonstrate a requirement for the IL-17/G-CSF signalling cascade in both neutrophil expansion and phenotype.

Next, we determined the source of IL-17. As T cells are known to produce IL-17 (refs 20, 21, 23), splenic CD3⁺ CD8⁺ T cells were analysed using a T-cell-specific gene expression array. This analysis validated upregulation of IL-17-related cytokines in T cells from tumour-bearing KEP mice (Extended Data Fig. 5f). We then asked whether lymphocytes are the only source of IL-17 and whether they drive metastasis. KEP mice were crossed with Rag1⁻/⁻ mice, which lack T and B cells. Tumour initiation, proliferation and histology were the same between KEP;Rag1⁻/⁻ and KEP;Rag1⁻/⁻ mice (Extended Data Fig. 6a and data not shown). However, KEP;Rag1⁻/⁻ mice exhibited lower levels of IL-17A and G-CSF in serum (Fig. 3d), decreased neutrophil counts (Fig. 3e) and altered neutrophil phenotype (Fig. 3f). Transforming growth factor β1 (TGF-β1) levels were unchanged between KEP;Rag1⁻/⁻ and KEP;Rag1⁻/⁻ mice (Extended Data Fig. 6b), suggesting that, unlike other models, TGF-β plays a lesser role in modulating neutrophil phenotype than IL-17-induced G-CSF. Importantly, KEP;Rag1⁻/⁻ mice displayed less pulmonary and lymph node metastases (Fig. 3g). The metastasis phenotype in KEP;Rag1⁻/⁻ mice was validated in the metastasis model...
where Rag1−/− mice were recipients of transplanted KEP tumour fragments, resulting in reduced pulmonary metastasis (Extended Data Fig. 6c). Thus, IL-17-producing lymphocytes drive neutrophil accumulation, phenotype and metastasis.

Direct ex vivo intracellular cytokine staining was performed to determine which T lymphocyte subset produces IL-17. Both CD4+ T cells and γδ T cells expressed IL-17A (Fig. 4a), and both IL-17-producing subpopulations were increased in various organs of tumour-bearing KEP mice compared with WT mice (Fig. 4b and Extended Data Fig. 7a). In primary tumours, the abundance of γδ and CD4+ T cells was too low (<0.2% and <2% of all live cells, respectively) to assess IL-17 expression reliably. γδ T cells exhibited higher IL-17A levels than CD4+ T cells (Fig. 4a and Extended Data Fig. 7b). Both cell populations were depleted to determine their functional importance. CD4+ T cell depletion lowered cKIT+ neutrophils, but failed to influence total neutrophil expansion, IL-17A or G-CSF levels (Extended Data Fig. 7c–e). Conversely, depletion of γδ T cells decreased IL-17A and G-CSF serum levels (Fig. 4c), reduced circulating neutrophils, lowered cKIT+ neutrophil proportions (Fig. 4d and Extended Data Fig. 7c) and reversed neutrophil phenotype (Fig. 4e). These data indicate that IL-17-producing γδ T cells promote neutrophil expansion and phenotypic alterations. IL-17-producing γδ T cells in tumour-bearing KEP mice were CD27−, mostly Vγ4+, and a proportion expressed CCR6, IL-1R1 and ROR-γT (Extended Data Fig. 8a, b) similar to other inflammatory diseases21.

We then asked how KEP mammary tumours activate IL-17-producing γδ T cells. On the basis of literature20–21 and cytokine analysis (Extended Data Fig. 5a, b), we focused on IL-23 and IL-1β. IL-17A expression by γδ T cells, G-CSF serum levels and neutrophil expansion was decreased by neutralization of IL-1β, but unaffected by inhibition of IL-23 (Fig. 4f–h). Macrophages were the most abundant IL-1β-expressing cell type in KEP tumours (Extended Data Fig. 8c, d). These data provide a mechanistic link between mammary tumours and γδ T cells.

Depletion of γδ T cells in the early phase of the metastasis model did not affect tumour histopathology, microvesSEL density or primary tumour

---

**Figure 3** | Lymphocyte-derived IL-17 is required for G-CSF-induced neutrophil expansion and phenotype. a. Cytokine levels in serum of WT (n = 5), tumour-bearing KEP mice (n = 9) and anti-IL-17- (n = 7) or anti-G-CSF-treated KEP mice (n = 6). b. Proportions of circulating neutrophils and cKIT-expressing neutrophils in KEP mice during primary tumour growth (n = 9 control, 8 anti-IL-17, 6 anti-G-CSF). c. Gene expression in circulating neutrophils from tumour-bearing KEP control mice (n = 9), anti-IL-17 (n = 6) or anti-G-CSF-treated KEP mice (n = 6). d. Cytokine levels in serum of tumour-bearing KEP;Rag1−/− (n = 9) and KEP;Rag1−/− mice (n = 7). e. Absolute blood neutrophil counts in tumour-bearing KEP;Rag1−/− mice (n = 8) or KEP;Rag1−/− mice (n = 5). f. Gene expression in circulating neutrophils from KEP;Rag1−/− (n = 10) and KEP;Rag1−/− mice (n = 8). g. Percentage of tumour-bearing mice with lung or lymph node metastasis (n = 50 KEP;Rag1−/−, 32 KEP;Rag1−/− mice). All data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 as determined by Mann–Whitney U-test or Fisher’s exact test.

**Figure 4** | IL-1β-activated, IL-17-producing γδ T cells regulate neutrophil expansion, neutrophil phenotype and metastasis. a. Intracellular staining within circulating T cells of tumour-bearing KEP mice. b. Proportion of IL-17A-producing γδ T cells (WT, n = 5; KEP, n = 6). c. Cytokine levels in serum of control (n = 10) and anti-γδTCR-treated (n = 7) KEP mice. d. Proportions of circulating neutrophils and cKIT-expressing neutrophils in KEP mice during primary tumour growth (n = 8 per group). e. Gene expression in circulating neutrophils from tumour-bearing KEP control mice (n = 10) and anti-γδTCR-treated KEP mice (n = 6). f. Proportion of IL-17A-producing γδ T cells in tumour-bearing KEP mice (n = 10 control, 5 anti-IL-23p19, 5 anti-IL-1β). g. Cytokine levels in serum (n = 9 KEP control, 5 anti-IL-23p19, 6 anti-IL-1β). h. Proportions of circulating neutrophils and cKIT-expressing neutrophils in KEP mice during primary tumour growth (n = 9 control, 5 anti-γδTCR-treated mice; n = 9 per group Tcrd−/− and Tcrd−/− mice). All data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 as determined by Mann–Whitney U-test or Fisher’s exact test.
growth (Extended Data Fig. 8e and data not shown). Importantly, however, pulmonary and lymph node metastases were significantly decreased in γδ T cell-depleted mice (Fig. 4i). These data were validated with Tcryd−/− mice, which lack γδ T cells. KEP tumour fragments were orthotopically transplanted into Tcryd−/− and Tcrd−/- mice and resected after outgrowth. Genetic elimination of γδ T cells also resulted in a significant reduction in pulmonary metastasis (Fig. 4j) without affecting primary tumour growth (Extended Data Fig. 8f). These data confirm a pro-metastatic role for γδ T cells.

In summary, we show that mammary tumour-induced, IL-17-producing γδ T cells drive systemic expansion and polarization of neutrophils towards a CD8+ T cell-suppressive phenotype and subsequent metastasis formation in distant organs (Extended Data Fig. 9). The importance of neutrophils during the early steps of the metastatic cascade and the upregulation of Prok2, S100a8 and S100a9 in neutrophils suggest that neutrophils may help to establish the pre-metastatic niche6.10.14; however, the role of these neutrophil-derived factors and others remains to be established in the KEP model. In patients with breast cancer, independent clinical studies consistently point towards a pro-metastatic role for neutrophils, γδ T cells and IL-17 (refs 3, 4, 26–29). Here, we establish a mechanistic connection between these independent clinical observations. In infection and inflammatory disorders, the γδ T cell/IL-17/neutrophil axis drives disease pathogenesis21,22,30. We now demonstrate that this targetable pathway also perpetuates breast cancer metastasis.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

**Received 11 April 2014; accepted 4 February 2015.**

**Published online 30 March; corrected online 17 June 2015 (see full-text HTML version for details).**

1. Quail, D. F. & Joyce, J. A. A microenvironmental regulation of tumor progression and metastasis. Nature Med. 19, 1423–1437 (2013).

2. McAllister, S. S. & Weinberg, R. A. The tumour-induced systemic environment as a critical regulator of cancer progression and metastasis. Nature Cell Biol. 16, 717–727 (2014).

3. Noh, H., Eomn, M. & Han, A. Usefulness of pretreatment neutrophil to lymphocyte ratio in predicting disease-specific survival in breast cancer patients. J. Breast Cancer 16, 55–59 (2013).

4. Azala, B. et al. Usefulness of the neutrophil-to-lymphocyte ratio in predicting short- and long-term mortality in breast cancer patients. Ann. Surg. Oncol. 19, 217–224 (2012).

5. Granot, Z. et al. Tumor entrained neutrophils inhibit seeding in the premetastatic lung. Cancer Cell 20, 300–314 (2011).

6. Kowanetz, M. et al. Granulocyte-colony stimulating factor promotes lung metastasis through mobilization of Ly6G+Ly6C+ granulocytes. Proc. Natl Acad. Sci. USA 107, 21248–21255 (2010).

7. Bajd, T. et al. Ultraviolet-radiation-induced inflammation promotes angiostomosis and metastasis in melanoma. Nature 507, 109–113 (2014).

8. Derksen, P. W. et al. Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. Cancer Cell 10, 437–449 (2006).

9. Doonmeab, C. W. et al. A preclinical mouse model of invasive lobular breast cancer metastasis. Cancer Res. 73, 353–363 (2013).

10. Kaplan, R. N. et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature 438, 820–827 (2005).

11. Effer, J. T. et al. Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. Cancer Cell 15, 35–44 (2009).

12. Kuonen, F. et al. Inhibition of the Kit ligand/c-Kit axis attenuates metastasis in a mouse model mimicking local breast cancer relapse after radiotherapy. Clin. Cancer Res. 18, 4365–4374 (2012).

13. Hiratsuka, S. et al. MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. Cancer Cell 2, 289–300 (2002).

14. Hiratsuka, S., Watanabe, A., Aburatani, H. & Maru, Y. Tumour-mediated upregulation of chemotactractants and recruitment of myeloid cells predetermine lung metastasis. Nature Cell Biol. 8, 1369–1375 (2006).

15. Pillay, J., Tak, T., Kamp, V. M. & Koenderman, L. Immune suppression by neutrophils and granulocytic myeloid-derived suppressor cells: similarities and differences. Cell. Mol. Life Sci. 70, 381–3827 (2013).

16. Mazzoni, A. et al. Myeloid suppressor line inhibit T cell responses by an NO-dependent mechanism. J. Immunol. 168, 689–695 (2002).

17. Young, M. R., Wright, M. A., Matthews, J. P., Malik, I. & Prechel, M. Suppression of T cell proliferation by tumor-induced granulocyte-macrophage progenitor cells producing transforming growth factor-β and nitric oxide. J. Immunol. 156, 1916–1922 (1996).

18. Lejeune, P. et al. Nitric oxide involvement in tumor-induced immunosuppression. J. Immunol. 152, 5077–5083 (1994).

19. Hamilton, J. A. & Achutmann, A. Colony stimulating factors and myeloid cell biology in health and disease. Trends Immunol. 34, 81–89 (2013).

20. Chung, Y. et al. Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. Immunity 30, 576–587 (2009).

21. Cai, Y. et al. Pivotal role of dermal IL-17-producing γδ T cells in skin inflammation. Immunity 35, 596–610 (2011).

22. Muz et al. Cxcr2 and Cxcl5 regulate the IL-17/G-CSF axis and neutrophil homeostasis in mice. J. Clin. Invest. 122, 974–986 (2012).

23. Sutton, C. E. et al. Interleukin-1 and IL-23 induce innate IL-17 production from gammamelta T cells, amplifying Th17 responses and autoimmunity. Immunity 31, 331–341 (2009).

24. Schwarzenberger, P. et al. Requirement of endogenous stem cell factor and granulocyte-colony-stimulating factor for IL-17-mediated granulopoiesis. J. Immunol. 164, 4783–4789 (2000).

25. Fridlender, Z. G. et al. Polarization of tumour-associated neutrophil phenotype by TGF-β: “N1” versus “N2” TAN. Cancer Cell 15, 183–194 (2009).

26. Han, Y. et al. Prognostic value of chemotherapy-induced neutropenia in early-stage breast cancer. Breast Cancer Res. Treat. 131, 483–490 (2012).

27. Ma, C. et al. Tumor-infiltrating γδ T lymphocytes predict clinical outcome in human breast cancer. J. Immunol. 185, 5029–5036 (2012).

28. Novotisky, S. V. et al. TGF-β1 receptor II loss promotes mammary carcinoma progression by Th17 dependent mechanisms. Cancer Discov 1, 430–441 (2011).

29. Chen, W. C. et al. Interleukin-17-producing cell infiltration in the breast cancer tumour microenvironment is a poor prognostic factor. Histopathology 63, 225–233 (2013).

30. Sutherland, T. E. et al. Chitinase-like proteins promote IL-17-mediated neutrophilia in a tradeoff between nematode killing and host damage. Nature Immunol. 15, 1116–1125 (2014).
METHODS

Mice. The generation and characterization of K14cre.Cdh1F/F;Trp53F/F (KEP) mice—a conditional model of invasive lobular breast cancer—has been described. KEP mice were backcrossed onto the FVB/N background. KEP mice were crossed with Rag1−/− mice (FVB/N; a gift from L. Courson) to generate KERP.Rag1−/− mice. The onset of mammary tumour formation was monitored twice weekly by palpation and calliper measurements starting at 4 months of age. Tera−/− mice on the FVB/N background were a gift from A. Hayday. The spontaneous metastasis model has also been described. Briefly, this model is based on the orthotopic transplantation of KEP tumour pieces into 10- to 12-week-old female recipient FVB/N mice, Rag1−/− mice, Tera−/− or Tera−/− mice. These tumour pieces are allowed to grow out, then surgically removed at 100 mm², after which 100% of mice develop overt metastatic disease. To deplete immune cells or neutralize cytokines, mice were injected intraperitoneally with an initial 400 µg followed by 100 µg thrice weekly for anti-Ly6G (clone 1A8; BioXCell), 200 µg twice weekly for anti-CD8 (clone 2.43; BioXCell) or 100 µg twice weekly for anti-γtCR (clone GL3; purified by the NKI facility). For cytokine neutralization experiments, KEP mice were injected intraperitoneally with 50 µg twice weekly for anti-IL-17A (clone 17F3; BioXCell), 50 µg thrice weekly for anti-G-CSF (clone 6D4/64; R&D Systems), 50 µg twice weekly anti-IL-23p19 (clone G23-8; BioXience) or 50 µg twice weekly anti-IL-1β (clone B12; BioXCell). Control animals were administered equal amounts of isotype control antibodies or equal volumes of PBS. Where indicated, WT and KEP mice were injected intraperitoneally with 5 µg rat rG-CSF (PEprotech) for four consecutive days and were killed on the fifth day. Tumour-bearing KEP mice injected with rG-CSF received anti-IL-17A at the same schedule as above. Antibody injections began when KEP mammary tumours reached 25 mm² until death at 225 mm², or transplanted tumours reached 9 mm² where indicated until surgery at 100 mm². Three independent KEP donor tumours were used throughout the remainder of the study. Blood samples were taken before and during antibody injections for flow cytometry analyses. Animals were randomized before beginning the treatment schedule. Mice were kept in individually ventilated and open cages, and food and water were provided ad libitum. Animal experiments were approved by the Animal Ethics Committee of the Netherlands Cancer Institute and performed in accordance with institutional, national and European guidelines for Animal Care and Use.

Immunohistochemistry. Formalin-fixed tissues were processed by routine procedures. Haematoxylin and eosin staining was performed as described. Citrate antigen retrieval was used for all staining procedures. Neutrophils were detected using either anti-Ly6B (clone 7/4; Cedarlane) or anti-Ly6G (clone 1A8; BD Biosciences), when primary tumours reached 100 mm². Quantitative analysis of neutrophil abundance was performed by counting cells in five high-power (>40) fields of view (FOV) per tissue. Metastases were detected using anti-cytokeratin 8 (clone Troma 1; Developmental Studies Hybridoma Bank, University of Iowa). In the metastasis model, the total number of cytokeratin 8+ nodules was scored in one lung section of each animal. The size of each nodule was measured using ImageJ, then represented as arbitrary units. Lymph node metastases were scored as positive or negative on the basis of the presence of cytokeratin 8+ metastases. Mouse that developed overt metastatic disease (that is, respiratory distress or 225 mm² axillary lymph nodes) was scored as positive. Lymph node metastases were scored as positive or negative. In the case of the presence of cytokeratin 8+ metastases. Stained slides were digitally processed in one lung 40) fields

Flow cytometry and intracellular staining. Tissues were collected in ice-cold PBS. Blood samples were collected in tubes containing heparin. Tumours and lungs were mechanically chopped using a McIlwain tissue chopper (Mickle Laboratory Engineering). Tumours were digested for 1 h at 37 °C in 3 mg ml⁻¹ collagenase A (Roche) and 25 µg ml⁻¹ DNase (Sigma) in serum-free DMEM medium. Lungs were digested for 30 min at 37 °C in 100 µg ml⁻¹ Liberase TM (Roche). Enzyme activity was neutralized by addition of cold DMEM/8% FCS and suspension was dispersed through a 70 µm cell strainer. Spleen, lymph nodes and liver were mashed through a 70 µm in one lung section of each animal. The size of each nodule was measured using ImageJ, then represented as arbitrary units. Lymph node metastases were scored as positive or negative on the basis of the presence of cytokeratin 8+ metastases. Stained slides were digitally processed using the Aperio ScanScope and captured using ImageScope software version 11.02. Brightness and contrast for representative images were adjusted equally among groups.

Flow cytometry and intracellular staining. Tissues were collected in ice-cold PBS. Blood samples were collected in tubes containing heparin. Tumours and lungs were mechanically chopped using a McIlwain tissue chopper (Mickle Laboratory Engineering). Tumours were digested for 1 h at 37 °C in 3 mg ml⁻¹ collagenase A (Roche) and 25 µg ml⁻¹ DNase (Sigma) in serum-free DMEM medium. Lungs were digested for 30 min at 37 °C in 100 µg ml⁻¹ Liberase TM (Roche). Enzyme activity was neutralized by addition of cold DMEM/8% FCS and suspension was dispersed through a 70 µm cell strainer. Spleen, lymph nodes and liver were mashed through a 70 µm in one lung section of each animal. The size of each nodule was measured using ImageJ, then represented as arbitrary units. Lymph node metastases were scored as positive or negative on the basis of the presence of cytokeratin 8+ metastases. Stained slides were digitally processed using the Aperio ScanScope and captured using ImageScope software version 11.02. Brightness and contrast for representative images were adjusted equally among groups.

Flow cytometry and intracellular staining. Tissues were collected in ice-cold PBS. Blood samples were collected in tubes containing heparin. Tumours and lungs were mechanically chopped using a McIlwain tissue chopper (Mickle Laboratory Engineering). Tumours were digested for 1 h at 37 °C in 3 mg ml⁻¹ collagenase A (Roche) and 25 µg ml⁻¹ DNase (Sigma) in serum-free DMEM medium. Lungs were digested for 30 min at 37 °C in 100 µg ml⁻¹ Liberase TM (Roche). Enzyme activity was neutralized by addition of cold DMEM/8% FCS and suspension was dispersed through a 70 µm cell strainer. Spleen, lymph nodes and liver were mashed through a 70 µm in one lung section of each animal. The size of each nodule was measured using ImageJ, then represented as arbitrary units. Lymph node metastases were scored as positive or negative on the basis of the presence of cytokeratin 8+ metastases. Stained slides were digitally processed using the Aperio ScanScope and captured using ImageScope software version 11.02. Brightness and contrast for representative images were adjusted equally among groups.
Real-time PCR. Neutrophil RNA was extracted as above then converted to complementary DNA (cDNA) with an AMV reverse transcriptase using Oligo(dT) primers (Invitrogen). cDNA (20 ng per well) was analysed by SYBR green real-time PCR with 500 nM primers using a LightCycler 480 thermocycler (Roche). β-actin was used as a reference gene. The following primer sequences were used for each gene: Nos2 forward 5’-GTTCTCAGCCAATAACAAGA-3’; reverse 5’-GTTGACGGGATGAGTCAC-3’; Prok2 forward 5’-CTTGGCTCTCTCTTCTCCT-3’; reverse 5’-GCATGTGCTGTGCTGTCAGT-3’; S100a8 forward 5’-TGGAGCACTCATTGCTCACC-3’; reverse 5’-ATGCCACACCCACTTTATCACC-3’; S100a9 forward 5’-GAAGAAAGAAGAGAAATGAAGCC-3’; reverse 5’-CTTTGCCATCAGCATCATACACTCC-3’; Il1b forward 5’-CAACCAGAAATGATATTCTCCATG-3’; reverse 5’-GATCCACACTCTCCAGCTGCA-3’; Actb forward 5’-CCTCATGAAGATCCTGACCGA-3’; reverse 5’-TTTGATGTCACGCACGATTTC-3’.

Fold change was calculated using the formula $2^{(\Delta \Delta C_t)}$.

T cell proliferation assay. Blood neutrophils from WT mice and splenic CD8+ T cells from WT mice were isolated by magnetic column (Miltenyi). Blood neutrophils from KEP mice with mammary tumours around 225 mm2 in size were also used. CD8+ T cells were labelled with Cell Trace CFSE following the manufacturer’s instructions (Invitrogen). Equal numbers of cells (2 × 10^5) were co-cultured in a 96-well flat bottom plate. CD3/CD28 Dynabeads (Invitrogen) were added according to manufacturer’s instruction, and the iNOS inhibitor, L-NMMA (Sigma), was added at 0.5 mM where indicated. After 48 h, T cell proliferation was evaluated on a BD LSRII flow cytometer using Diva software using the following antibodies: CD8a-PE (1:600; clone 53-6.7), CD11b-APC (1:400; clone M1/70), Ly6C-eFluor 450 (1:400; clone HK1.4), Ly6G-AlexaFluor 700 (1:400; clone 1A8) and 7AAD viability marker. Data analyses used FlowJo Software version 9.7.1. Proliferation index was calculated using the formula [(percentage of proliferated, co-cultured CD8+ T cells)/(percentage of proliferated CD8+ T cells without co-culture)] × 100, for each replicate experiment.

Cytokine analysis. Multiplex quantification of cytokines and chemokines in mammary glands and tumours was performed using the premixed 24-plex Bio-Plex Pro Mouse Cytokine Assay (Bio-Rad) according to the manufacturer’s recommendations. Protein lysates were prepared as previously described. Unsupervised clustering was performed on normalized, median-centred data then converted to a heat-map using Genesis software. For IL-17A and G-CSF serum levels, BD Cytometric Bead Arrays were used as directed and analysed on a Cyan flow cytometer with Summit software (Beckman Coulter). Data analyses used FlowJo Software version 9.7.1. For TGF-β1, a DuoSet ELISA kit was purchased from R&D Systems and performed according to the manufacturer’s instructions.

PCR array. The spleens of three WT or KEP mammary tumour-bearing mice were pooled and labelled with anti-CD3 antibodies. CD3+ T cells were sorted using a BD FACSAria II. RNA was isolated with Trizol as above. Gene expression differences were analysed using a mouse T cell-specific PCR array from Qiagen according to their instructions and software. Genes exhibiting a threefold change were considered biologically relevant.

Statistical analysis. Data analyses used GraphPad Prism version 7. Applied analyses are indicated in corresponding legends. No statistical methods were used to predetermine sample sizes. Sample sizes were based on previous experience with the models. Differences with $P < 0.05$ were considered statistically significant.

31. de Visser, K. E., Korets, L. V. & Coussens, L. M. De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. Cancer Cell 7, 411–423 (2005).
32. Ciampricotti, M., Hau, C. S., Doornebal, C. W., Jonkers, J. & de Visser, K. E. Chemotherapy response of spontaneous mammary tumors is independent of the adaptive immune system. Nature Med. 18, 344–346 (2012).
33. Girardi, M. et al. Regulation of cutaneous malignancy by γδ T cells. Science 294, 605–609 (2001).
34. Ciampricotti, M. et al. Development of metastatic HER2+ breast cancer is independent of the adaptive immune system. J. Pathol. 224, 56–66 (2011).
Extended Data Figure 1 | Systemic neutrophil expansion and accumulation in mammary tumour-bearing KEP mice and the metastasis model.

a, Representative images of neutrophils identified by the 7/4 antibody in lung sections in WT or KEP mice. Scale bar, 50 μm. 
b, Quantification of neutrophil accumulation per field of view (FOV) in various organs by immunohistochemistry using the 7/4 antibody (n = 6 WT, 9 KEP mice).
c, Absolute neutrophil counts in blood of WT and tumour-bearing KEP mice (n = 4 WT, 8 KEP).
d, Quantification of neutrophil accumulation in various organs determined by flow cytometry and gated on CD45⁺ cells. Neutrophils were not detectable in WT mammary glands (n = 5 WT, 7 KEP mice).
e, Representative images of Ly6G-stained lung sections and quantification of neutrophil accumulation in the metastasis model. Data were generated from mock-transplanted, non-tumour-bearing mice (0 mm²), or tumour-transplanted recipient mice killed when tumours reached the tumour size shown or when mice exhibited signs of respiratory distress due to pulmonary metastasis. For quantification in lungs with metastases, neutrophils residing inside metastases were excluded. 
f, Pulmonary metastatic lesion. Scale bar, 100 μm (n = 3, 5, 6, 6 and 3 mice for 0, 9, 25, 100 mm² and metastasis respectively). 
g, Kinetics of neutrophil accumulation in various organs of the metastasis model by flow cytometry after gating on CD45⁺ cells. Recipient mice transplanted with KEP tumour pieces were killed at the tumour size shown (n = 6, 5, 6, and 7 mice for 0, 9, 25, 100 mm² respectively). 
g, Kinetics of neutrophil proportions in blood (gated on CD45⁺ cells), before and after surgical removal of their primary tumour (n = 5). All data are mean ± s.e.m. 
*P < 0.05, **P < 0.01, ***P < 0.001 as determined by Mann–Whitney U-test or Kruskal–Wallis test followed by post-hoc Dunn’s test.
Extended Data Figure 2 | Neutrophil depletion does not affect primary tumour or metastatic nodule growth. a, Schematic illustration of the neutrophil depletion experiment in the spontaneous metastasis model. b, Representative dot plots of neutrophils gated on CD45+ cells in blood of control and anti-Ly6G-treated recipient mice. The Gr1 antibody was used here to avoid false-negative results since the anti-Ly6G depleting antibody may mask the Ly6G epitope. CD11b+Gr1high cells were Ly6C−CCR2−, indicating that these cells were neutrophils. CD11b+Gr1low cells that were Ly6C+ and CCR2+ represented the monocytic fraction. c, Quantification of neutrophil depletion in blood of control and anti-Ly6G-treated recipient mice at the tumour size indicated (n = 8 control, 5 anti-Ly6G; **P < 0.01 as determined by Mann–Whitney U-test). d, Primary tumour growth kinetics of mice treated as indicated (n = 12 control, 14 anti-Ly6G). e, Representative images of primary tumours in the metastasis model treated as shown and stained with haematoxylin and eosin, cytokeratin 8, vimentin, E-cadherin and CD34. Scale bar, 100 μm. f, Quantification of blood vessels per field of view (FOV) in control and neutrophil-depleted mice by anti-CD34 immunohistochemistry (n = 10). g, Quantification of pulmonary metastatic nodule size in control and neutrophil-depleted mice (n = 9 control, 11 early phase, 14 late phase mice). All data are mean ± s.e.m.
Extended Data Figure 3 | Subpopulations of neutrophils in mammary tumour-bearing mice are immature. a, Gating strategy for identification of neutrophils (CD45⁺CD11b⁺Ly6G⁺Ly6C⁻F4/80⁻ cells), cKIT⁺ neutrophils and monocytes (CD45⁺CD11b⁺Ly6G⁻Ly6C⁺F4/80⁻ cells) by flow cytometry. Blood cells from WT and tumour-bearing KEP mice are shown here. b, Quantification of cKIT⁺ neutrophil accumulation in various organs determined by flow cytometry after gating on CD45⁺CD11b⁺Ly6G⁺Ly6C⁻F4/80⁻ cells. cKIT⁺ neutrophils were not detectable in WT mammary glands (n = 5 WT, 7 KEP; Mann–Whitney U-test). c, cKIT⁺ neutrophil proportions in various organs of the metastasis model as determined by flow cytometry after gating on CD45⁺CD11b⁺Ly6G⁺Ly6C⁻F4/80⁻ cells. Mice were killed at the tumour size shown (n = 5, 5, 5, and 8 mice for 0, 9, 25, 100 mm² respectively; Kruskal–Wallis test followed by post-hoc Dunn’s test). d, Kinetics of cKIT⁺ neutrophil proportions in blood (gated on CD45⁺CD11b⁺Ly6G⁺Ly6C⁻F4/80⁻ cells), before and after surgical removal of the primary tumour (n = 5 per group; Mann–Whitney U-test). e, Representative images and quantification of neutrophil nuclear morphology. Ly6G⁺ cells were isolated from blood of WT and tumour-bearing KEP mice then assessed by Giemsa stain. Hyper-segmented cells were considered mature, whereas all other cells were considered immature. Scale bar, 10 μm. (n = 6 WT, 5 KEP mice; Mann–Whitney U-test). All data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001.
Extended Data Figure 4 | Neutrophils influence the function and phenotype of CD8+ T cells. a, Unsupervised hierarchical clustering of RNA-Seq analysis depicting 100 differentially expressed genes between circulating neutrophils from WT and tumour-bearing KEP mice. P value (0.05) was used as cutoff (n = 4 WT, 5 KEP mice). See also Extended Data Table 1 for top 50 genes ranked by fold change. b, Circulating neutrophils from either WT or tumour-bearing KEP mice were incubated with CFSE-labelled splenic CD8+ T cells from WT mice and CD3/CD28 stimulation beads. The iNOS inhibitor, L-NMMA, was added where indicated. After 48 h, CD8+ T cell proliferation was measured by flow cytometry. c, Dot plots depicting live cell-gated CD8+ T cell proportions in lungs of mice in control and neutrophil-depleted mice, killed when transplanted tumours reached 100 mm². d, Dot plots of effector CD8+ T cell (CD62L−CD44+) proportions in lungs of transplanted mammary tumour-bearing mice that were killed when tumours reached 100 mm². e, IFN-γ expression by CD8+ T cells in lungs of transplanted mammary tumour-bearing mice that were killed when tumours reached 100 mm². f, Tumour growth kinetics in neutrophil-depleted or combined neutrophil- and CD8+ T-cell-depleted, mammary tumour-transplanted recipient mice, compared with control (n = 13 control, 21 anti-Ly6G, 14 anti-Ly6G/CD8). Data are mean ± s.e.m.
Extended Data Figure 5 | Cytokine expression levels in tumours and T cells, and their effects on neutrophils. 

a, Unsupervised clustering of cytokine expression analysis in WT mammary glands and KEP tumours. Protein lysates were prepared as previously described from whole tissue and analysed for expression of various cytokines by Luminex-based assay (n = 5 per group).

b, Protein levels of indicated cytokines in WT mammary glands and KEP tumours, determined by Luminex-based cytokine profiling; n.d., not detectable (n = 10 per group; Mann–Whitney U-test).

c, d, Quantification of neutrophil and cKIT-expressing neutrophil accumulation in blood as determined by flow cytometry and gated on CD45+ cells. WT (n = 4) or tumour-bearing KEP mice (n = 9) were treated with anti-IL-17 (n = 8) and/or recombinant G-CSF (rG-CSF; n = 4) where indicated (Mann–Whitney U-test or Kruskal–Wallis test followed by post-hoc Dunn’s test).

e, Gene expression in circulating neutrophils from WT control (n = 5), rG-CSF-treated WT mice (n = 4), KEP control (n = 10), anti-IL-17-treated (n = 6), anti-IL-17 + rG-CSF-treated KEP mice (n = 4; Mann–Whitney U-test or Kruskal–Wallis test followed by post-hoc Dunn’s test). Spleens of three WT mice and three KEP mice were pooled and CD3+ T cells were isolated. These cells were analysed by a real-time PCR array containing 86 different genes. Gene expression changes greater than threefold are shown. Members of the IL-17 signalling pathway are depicted in blue. *P < 0.05, **P < 0.01, ***P < 0.001. All data are mean ± s.e.m.
Extended Data Figure 6 | Absence of the adaptive immune system reduces metastasis. a, Graphic representation of mammary tumour latency (left) and tumour growth (right) in lymphocyte-proficient KEP;Rag11/−/− and lymphocyte-deficient KEP;Rag12/−/− mice (n = 30 per group left panel, 10 mice per group right panel). b, Levels of TGF-β1 in mammary tumours and the plasma of tumour-bearing mice (n = 6 tumour, 3 plasma). c, Quantification of metastatic burden in lungs of recipient WT or Rag11/−/− mice that were transplanted with KEP mammary tumour fragments and underwent surgical removal of the primary tumour (n = 6 WT, 4 Rag11/−/− mice; **P < 0.01, Mann–Whitney U-test). Data are mean ± s.e.m.
Extended Data Figure 7 | Depletion of CD4+ T cells does not affect systemic cytokine levels or neutrophil expansion. 

a, The proportion of IL-17A+ cells among CD4+ T cells in organs of WT and tumour-bearing KEP mice (n = 6 per group; Mann–Whitney U-test). 
b, Median fluorescence intensity of IL-17A expression in circulating γδ and CD4+ T cells from tumour-bearing KEP mice, as determined by flow cytometry (n = 11 per group; Wilcoxon matched-pairs test). 
c, Representative dot plots depicting total neutrophil and cKIT+ proportions in blood of control, anti-CD4- and anti-γδTCR-treated tumour-bearing KEP mice. 
d, Quantification of total neutrophil and cKIT+ neutrophil proportions in blood of control and anti-CD4-treated tumour-bearing KEP mice (n = 7 per group; Mann–Whitney U-test). 
e, Serum levels of IL-17A and G-CSF in control and anti-CD4-treated tumour-bearing KEP mice (n = 10 control, 6 anti-CD4; Mann–Whitney U-test). *P < 0.05, **P < 0.01. All data are mean ± s.e.m.
Extended Data Figure 8 | γδ T cell phenotype in KEP mice and their lack of influence on tumour growth in the metastasis model. a, γδ T cells from lungs of tumour-bearing KEP mice were analysed by flow cytometry for IL-17, CD27, Vγ1 and Vγ4 expression. Two major populations of γδ T cells were observed including IL-17^+ CD27^- and IL-17^- CD27^- cells. b, Representative histograms of CCR6, IL-1R1, IL-23R and ROR-γt expression in IL-17^- CD27^- and IL-17^- CD27^- γδ T cell populations shown in a. c, Il1b gene expression in various cell populations isolated from transplanted KEP tumours. Cells from three mice were pooled to form one group. CD45^- cells (which include cancer cells, endothelial cells and fibroblasts), CD45^- CD11b^- F4/80^- macrophages, CD45^- CD11b^- Ly6G^- neutrophils and CD45^- CD11b^- lymphocytes were sorted from two pooled groups. Real-time PCR was performed on individual cell populations for Il1b expression. Relative expression among different cells is shown. d, Graphic representation of immune cell proportions in KEP tumours (n = 4). e, Primary tumour growth kinetics of control and γδ T cell-depleted tumour transplant recipient mice (n = 13 per group). f, Growth kinetics of primary tumours transplanted into Tcrδ^-/- (n = 10) and Tcrδ^-/- mice (n = 6). All data are mean ± s.e.m.
Extended Data Figure 9 | The γδ T cell/IL-17/neutrophil axis promotes breast cancer metastasis. Mammary tumours evoke a systemic inflammatory cascade that is initiated by IL-1β production. Tumour-derived IL-1β activates γδ T cells to produce IL-17. Increased systemic IL-17 levels lead to upregulation of G-CSF, which subsequently causes neutrophil expansion and alteration of neutrophil phenotype. These phenotypically altered neutrophils produce iNOS that suppresses the activity of anti-tumour CD8⁺ T cells. As a result of this systemic inflammatory cascade, the chance that disseminated cancer cells can establish metastases in distant organs is maximized.
Extended Data Table 1  | The top 50 most differentially expressed genes between neutrophils from WT and KEP mice

| Gene ID | Gene name                  | Ensembl gene ID      | fold change | p value |
|---------|----------------------------|----------------------|-------------|---------|
| Nos2    | nitric oxide synthase 2, inducible | ENSMUSG00000020826 | 31.5        | 0.345   |
| Car4    | carbonic anhydrase 4        | ENSMUSG0000000805   | 26.5        | 0.111   |
| Lipg    | lipase, endothelial        | ENSMUSG00000053846  | 24.6        | 0.128   |
| Gm11430 | predicted gene 11430       | ENSMUSG00000080927  | 16.7        | 0.292   |
| Gm6551  | predicted gene 6551        | ENSMUSG00000078100  | 14.2        | 0.039   |
| Sfpa3   | stefin A3                  | ENSMUSG00000054905  | 14.1        | 0.047   |
| Pvr2    | poivovirus receptor-related 2 | ENSMUSG00000062300 | 13.8        | 0.143   |
| Gm16748 | predicted gene, 16748      | ENSMUSG00000085308  | 13.5        | 0.087   |
| Prok2   | prokinetin 2               | ENSMUSG00000030069  | 12.3        | 0.024   |
| Esm1    | endothelial cell-specific molecule 1 | ENSMUSG00000042379 | 12.1        | 0.010   |
| Ano2    | anoctamin 2                | ENSMUSG00000038115  | 10.6        | 0.138   |
| Saa1    | serum amyloid A 1          | ENSMUSG00000074115  | 10.1        | 0.192   |
| Nov     | nephroblastoma overexpressed gene | ENSMUSG00000037352 | 9.6         | 0.295   |
| Gpr15   | G protein-coupled receptor 15 | ENSMUSG00000047293 | 9.5         | 0.204   |
| Ggt1    | gamma-glutamyltransferase 1 | ENSMUSG00000060345 | 6.3         | 0.076   |
| Cish    | cytokine inducible SH2-containing protein | ENSMUSG00000032578 | 9.1         | 0.217   |
| Sfpa2   | stefin A2                  | ENSMUSG00000022902  | 8.9         | 0.020   |
| Gm14028 | predicted gene 14028       | ENSMUSG00000082339  | 8.4         | 0.036   |
| Sfpa1   | stefin A1                  | ENSMUSG00000071562  | 7.5         | 0.087   |
| Cht1    | complement factor H-related 1 | ENSMUSG00000057037 | 7.1         | 0.029   |
| Ms4a3   | membrane-spanning 4-domains, subfamily A, member 3 | ENSMUSG00000024681 | 6.8         | 0.177   |
| Kit     | kit oncogene               | ENSMUSG00000005672  | 6.8         | 0.085   |
| Jphp3   | junctophilin 3             | ENSMUSG00000025318  | 6.8         | 0.076   |
| Cnnm2   | cyclin M2                  | ENSMUSG00000064105  | 6.6         | 0.128   |
| Gnb5    | guanine nucleotide binding protein (G protein), beta 5 | ENSMUSG00000032192 | 6.4         | 0.004   |
| Alox12   | arachidonate 12-lipoxygenase | ENSMUSG00000003320 | -11.2       | 0.103   |
| Sfrn14-ps | schlafen family member 14, pseudogene | ENSMUSG00000082101 | -11.2       | 0.049   |
| Gm6634  | predicted gene 6634        | ENSMUSG00000086538  | -11.3       | 0.139   |
| Syl13   | synaptotagmin XIII         | ENSMUSG00000027220  | -11.4       | 0.213   |
| Tsc2d1  | TSC22 domain family, member 1 | ENSMUSG00000022010 | -11.4       | 0.107   |
| Gm10419 | predicted gene 10419       | ENSMUSG00000072799  | -11.5       | 0.231   |
| Sh3bg2r2 | SH3 domain binding glutamic acid-rich protein like 2 | ENSMUSG00000032205 | -11.6       | 0.117   |
| Fhl1    | four and a half LIM domains 1 | ENSMUSG00000023092 | -11.9       | 0.131   |
| Trpc6   | transient receptor potential cation channel, subfamily C, member 6 | ENSMUSG00000031997 | -11.9       | 0.076   |
| Cilla2a  | cytotoxic T lymphocyte-associated protein 2 alpha | ENSMUSG00000044258 | -12.0       | 0.107   |
| Csgalnt1 | chondroitin sulfate N-acetylgalactosaminyltransferase 1 | ENSMUSG00000036356 | -12.2       | 0.156   |
| Gng11   | guanine nucleotide binding protein (G protein), gamma 11 | ENSMUSG00000032766 | -12.2       | 0.123   |
| 2610109H07Rik | RIKEN cDNA 2610109H07 gene | ENSMUSG00000029005 | -12.3       | 0.195   |
| Nrgn    | neurogranin                | ENSMUSG00000053310  | -12.5       | 0.087   |
| Gm11274 | predicted gene 11274       | ENSMUSG00000056311  | -12.6       | 0.101   |
| Peg10   | paternally expressed 10    | ENSMUSG00000092035  | -13.1       | 0.177   |
| Angpt1  | angiopoietin 1             | ENSMUSG00000022309  | -13.8       | 0.168   |
| Plp1    | proteolipid protein (myelin) 1 | ENSMUSG00000031425 | -13.8       | 0.132   |
| Dlg2    | discs, large homolog 2 (Drosophila) | ENSMUSG00000052572 | -14.6       | 0.080   |
| Syt4    | synaptotagmin-like 4       | ENSMUSG00000031255  | -14.6       | 0.138   |
| Mpl     | myeloproliferative leukemia virus oncogene | ENSMUSG00000068389 | -15.6       | 0.197   |
| Mras    | muscle and microspikes RAS | ENSMUSG00000032470 | -15.7       | 0.053   |
| Gp6     | glycoprotein 6 (platelet)  | ENSMUSG00000078810  | -15.9       | 0.215   |
| Cd226   | CD226 antigen              | ENSMUSG00000034028  | -18.4       | 0.134   |
| Bean1   | brain expressed, associated with Nedd4, 1 | ENSMUSG00000031872 | -19.2       | 0.053   |