Regulation of antitumour CD8 T-cell immunity and checkpoint blockade immunotherapy by Neuropilin-1

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Neuropilin-1 (Nrp-1) is a marker for murine CD4⁺FoxP3⁺ regulatory T (Treg) cells, a subset of human CD4⁺ Treg cells, and a population of CD8⁺ T cells infiltrating certain solid tumours. However, whether Nrp-1 regulates tumour-specific CD8 T-cell responses is still unclear. Here we show that Nrp-1 defines a subset of CD8⁺ T cells displaying PD-1hi status and infiltrating human lung cancer. Interaction of Nrp-1 with its ligand semaphorin-3A inhibits migration and tumour-specific lytic function of cytotoxic T lymphocytes. In vivo, Nrp-1⁺PD-1hi CD8⁺ tumour-infiltrating lymphocytes (TIL) in B16F10 melanoma are enriched for tumour-reactive T cells exhibiting an exhausted state, expressing Tim-3, LAG-3 and CTLA-4 inhibitory receptors. Anti-Nrp-1 neutralising antibodies enhance the migration and cytotoxicity of Nrp-1⁺PD-1hi CD8⁺ TIL ex vivo, while in vivo immunotherapeutic blockade of Nrp-1 synergises with anti-PD-1 to enhance CD8⁺ T-cell proliferation, cytotoxicity and tumour control. Thus, Nrp-1 could be a target for developing combined immunotherapies.
Cytotoxic T lymphocytes (CTL), predominantly expressing T-cell co-receptor CD8, play a major role in the anti-tumour immune response. To destroy malignant cells, CTL must first migrate to the tumour site, infiltrate tumour cell clusters, and then interact with malignant cells to achieve their cytotoxic functions after T-cell receptor (TCR) recognition of specific tumour peptide-major histocompatibility complex class I (MHC-I) complexes on target cells. To perform this killing function, activated CTL are armed with various effector molecules, including pro-inflammatory cytokines, in particular IFNγ and TNF, and cytotoxic granules containing perforin and granzymes. However, cancer cells frequently escape CD8 T-cell recognition and reactivity. A decrease in cell surface expression of peptide/MHC-I complexes on cancer cells is one mechanism involved in this lack of functional activity. However, it now appears clear that engagement of inhibitory receptors such as CTLA-4, PD-1 and Tim-3, expressed on CD8+ tumour-infiltrating lymphocytes (TIL) with their respective ligands on target cells, is another critical constraint explaining the poor reactivity of these cells in the tumour microenvironment. Moreover, PD-1 expression on CD8+ TIL seems to be a characteristic of donally expanded CD8+ tumour-reactive T cells identified in cancer patients.

In this context, elucidating the mechanisms of CTLA-4 and PD-1 T-cell inhibitory signalling has led to development of promising cancer immunotherapy tools, including blocking monoclonal antibodies (mAb) targeting these so-called ‘immune checkpoints’. Yet, to be efficient, immune checkpoint blockade therapies require strong tumour infiltration by CTL whose activities are subjected to such inhibition. Indeed, the therapeutic benefit of the PD-1 blockade requires that tumours be infiltrated by CD8+ TIL strongly expressing the PD-1 receptor. Under these conditions, CD8+ T-cell responses to tumour-specific antigens parallel tumour regression, and appear to be directly associated with clinical benefits of anti-PD-1 immunotherapy. Unfortunately, only a fraction of cancer patients respond to PD-1 blockade and, among long-term responders, relapses are often observed after initial tumour regression. In this regard, a high proportion of tumours, referred to as immune deserts or cold tumours, are not infiltrated by immune cells and are thus poorly responsive to immunotherapy. Moreover, additional as yet undescribed inhibitory mechanisms inherent to CD8+ TIL and leading to tumour resistance likely exist.

In this light, we aimed to analyse the role of neuropilin-1 (Nrp-1) during antitumour CD8+ T-cell responses. Nrp-1 acts as a co-receptor for extracellular ligands, including secreted members of the class 3 semaphorin (Sema-3) family, isoforms of vascular endothelial growth factor (VEGF) and transforming growth factor β. This transmembrane glycoprotein is involved in axon guidance and neuronal development. More recently, a growing body of evidence suggests that Nrp-1 has multiple roles in the immune system and in T-cell responses. In mice, Nrp-1 is expressed at high levels on natural regulatory T cells, and its expression by Treg cells is responsible for reduced antitumour immunity. Nrp-1 deficiency on murine FoxP3+ CD4+ Treg cells has been reported to delay tumour growth correlated with enhanced activation of tumour-infiltrating CD8+ T cells. In humans, Nrp-1 is also expressed on a subset of CD4+ Treg cells in lymph nodes and on CD4+ TIL, including suppressive Treg. However, much less is known about the expression of Nrp-1 on CD8+ TIL and its involvement in regulating their functions. Nrp-1 has been found to be expressed by murine-tolerant self-reactive CD8+ T cells, and by both mouse and human melanoma-infiltrating CD8+ T lymphocytes. Nevertheless, expression of Nrp-1 on CD8+ TIL has not been thus far systematically addressed, and its potential role in regulating CTL activities and inhibiting antitumour T-cell functions is unknown.

Here we show that Nrp-1 is expressed on a subset of human CD8+ TIL in non-small-cell lung cancer (NSCLC) tumours and on murine CD8+ TIL from several tumour types. These T lymphocytes express high levels of PD-1, defining a subset of Nrp-1+PD-1hi TIL enriched with tumour-specific T cells. Anti-Nrp-1 blocking mAb restores CD8+ T-cell effector functions and optimise suppression of tumour progression induced by anti-PD-1. We conclude that Nrp-1 is an immune checkpoint that negatively regulates antitumour CD8+ T-cell immunity, and is thus a promising target for combination cancer immunotherapies.

Results

Nrp-1 is expressed on a subset of CD8+ TIL in human NSCLC. Nrp-1 is expressed on CD4+ Treg cells in human lymph nodes and in TIL from colorectal cancer metastases. However, little is known about its expression on CD8+ TIL. We first evaluated the expression of NRPI transcripts in primary human lung tumours and autologous normal lungs. Quantitative real-time PCR (qRT-PCR) showed high expression levels of NRPI mRNA in some lung tumour samples compared with the cognate normal lung (Supplementary Fig. 1a). The NSCLC tumour sample 8 was found to display a high increase in Nrp-1 mRNA expression, and NSCLC samples 1, 2 and 4 were found to display about a two fold expression increase compared with autologous healthy lungs. Human NSCLC were also found to express NRP2, as tumour samples 1, 3 and 8 (Supplementary Fig. 1a), and certain Plexin (Plxn) A or D family member transcripts (Supplementary Fig. 1b), as well as genes encoding Nrp ligands of the Sema-3 family, such as Sema-3B, -3C, -3E and -3F (Supplementary Fig. 1c). At the protein level, we also found an expression of Nrp-1 on some epithelial tumour cells from freshly dissociated human NSCLC and on all the tested lung tumour cell lines (Supplementary Fig. 2a, b). These results were consistent with previous studies having shown that malignant cells frequently expressed Nrp-1, and in which Nrp-1 expression was associated with tumour progression.

We next focused on the expression of Nrp-1 protein in freshly isolated TIL. NSCLC tumours from 28 patients were analysed. Immunofluorescence analyses showed that Nrp-1 was expressed on a subset of human lung CD3+ TIL. In contrast, parallel analysis of peripheral blood lymphocytes (PBL) from lung cancer patients and healthy donors (HD) showed that circulating T cells did not express the receptor (Fig. 1a). Expression of Nrp-1 was observed on both CD8+ and CD4+ TIL, but with a higher frequency on CD8+ TIL (14.2 ± 2.1% vs 8.4 ± 0.9%). Moreover, expression of Nrp-1 correlated with the activation state of T lymphocytes, as it was more frequent on CD25+ and PD-1+ T cells from both CD8+ and CD4+ TIL subsets than on CD25− and PD-1− subsets (Fig. 1b, c). Indeed, 70.1 ± 8.3% of Nrp-1−CD8+ TIL and 76.9 ± 4.2% of Nrp-1+CD4+ TIL also expressed high levels of PD-1 (PD-1hi). Consistently, activation of HD PBL with immobilised anti-CD3 mAb induced expression of the protein (Supplementary Fig. 2c, d). In contrast, no obvious correlation between Nrp-1 and FoxP3 expression (p = 0.2578) was observed in CD4+ TIL, because equal percentages of Nrp-1− T cells were found in both FoxP3+ and FoxP3− subsets, which showed similar percentages of PD-1hi cells (Fig. 1d, Supplementary Table 1). These results show that Nrp-1 is expressed on a subset of activated human CD4+ and CD8+ TIL displaying PD-1hi status in NSCLC tumours.

Binding of Sema-3A to human Nrp-1 impairs T-cell functions. Sema-3A, a secreted member of the Sema-3 family, is a
As human CD8+ TIL expressed substantial amounts of Nrp-1, this raises the question as to whether its interaction with Sema-3A contributes to the T-cell dysfunction often observed in the tumour microenvironment. To evaluate this hypothesis, we first used the IGR-Pub lung adenocarcinoma cell line and autologous CTL clone P62 established from TIL of a NSCLC patient\textsuperscript{24}. Initial studies indicated that the IGR-Pub cell line, as well as fresh NSCLC tumours and several other human lung cancer cell lines, and to a lesser extent, normal bronchial epithelial cell line 16HBE, produced Sema-3A.

**Fig. 1** Expression of Nrp-1 and PD-1 on T cells infiltrating human NSCLC tumours. a Surface expression of Nrp-1 on CD4+ and CD8+ T cells from TIL and PBL of NSCLC patients, and compared with PBL from HD. Percentages of positive cells are indicated. Right: percentages of Nrp-1 on CD8+ and CD4+ T cells in TIL (n = 28) and PBL from NSCLC patients (n = 11) and HD (n = 12). b Expression of Nrp-1 in CD25 and PD-1 CD8+ T cells subsets from NSCLC TIL. Percentages of Nrp-1 among CD8+ TIL expressing or not CD25 (n = 16) or PD-1 (n = 13). Right: dot plot showing co-expression of Nrp-1 and PD-1 on CD8+ TIL from one representative patient. c Expression of Nrp-1 in CD25 and PD-1 CD4+ T cells subsets from NSCLC TIL. Percentages of Nrp-1 among CD4+ TIL expressing or not CD25 (n = 20) or PD-1 (n = 12). Right: dot plot showing co-expression of Nrp-1 and PD-1 on CD4+ TIL from one representative patient. d Expression of Nrp-1 in conventional and regulatory CD4+ T cells subsets. Percentages of Nrp-1 among CD4+ TIL expressing or not FoxP3 (n = 9). Right: dot plot showing co-expression of Nrp-1 and PD-1 on CD4+ TIL expressing or not FoxP3 from one representative patient. Means ± SEM one-way ANOVA test with Bonferroni correction a or two-tailed Student’s unpaired t test b, c, d. *P < 0.05; **P < 0.01; ***P < 0.001.
Fig. 2 Expression of Sema-3A and Nrp-1 in human lung tumour cell lines and CTL clone. 

a Sema-3A expression in human lung tumour cell lines. Total protein extracts were analysed by western blot using anti-Sema-3A mAb. The 16HBE cell line was included as a control. Full length and proteolytically processed proteins are indicated.

b Co-expression of Nrp-1 and CD25, and Nrp-1 and PD-1 on P62 T cells stimulated with immobilised anti-CD3. The P62 clone was unstimulated or stimulated with anti-CD3, pre-incubated with Sema-3A-Fc, and then labelled with anti-human IgG-Fc secondary mAb. Results are gMFI mean ± SEM of triplicate samples.

c Sema-3A-Fc inhibits CTL migration toward a CXCL12 gradient. The T-cell clone was stimulated with anti-CD3, pre-incubated with BSA or Sema-3A-Fc, and then seeded in the upper chambers of transwell plates and exposed to a gradient of CXCL12 loaded in the lower chambers. The number of T cells that had migrated into the lower chambers was determined. Results are mean chemotaxis index ± SEM of triplicate samples.

d Sema-3A-Fc inhibits CD8+ TIL migration toward CXCL12. CD8+ TIL isolated from three human NSCLC tumours were stimulated with anti-CD3, pre-incubated with BSA or Sema-3A-Fc, and then seeded in the upper chambers of transwells and exposed to a CXCL12 gradient. Results are mean chemotaxis index ± SEM of triplicates.

e Cytotoxicity of the CTL clone toward autologous tumour cells. The P62 clone was stimulated with anti-CD3, pre-incubated in medium or with Sema-3A-Fc. Cytotoxicity toward the cognate IGR-Pub cell line was determined.

f Cytotoxic activity of freshly isolated TIL toward autologous tumour cells. TIL, freshly isolated from a NSCLC tumour, were stimulated with anti-CD3, pre-incubated in medium or with Sema-3A-Fc. Cytotoxicity toward freshly isolated autologous tumour cells was determined. Data shown for cytotoxic assay correspond to one of three independent experiments. Means ± SEM two-tailed Student’s paired t test c, one-way ANOVA test with Bonferroni correction d, e or two-way ANOVA test with Bonferroni correction f, g. *P < 0.05; **P < 0.01; ***P < 0.001. Source data are provided as a Source Data file.
as detected by western blot (Fig. 2a) and/or intracellular immunofluorescence staining (Supplementary Fig. 3a, b). Human fresh NSCLC tumours and tumour cell lines also expressed Sema-3F, another ligand of Nrp, as detected by intracellular staining (Supplementary Fig. 3c, d). In contrast, the expression of Nrp-1 on the P62 T-cell clone membrane varies according to its activation state with feeder cells and IL-2. Consistently, stimulation with plastic-coated anti-CD3 mAb induced strong Nrp-1 expression on the surface of P62 T cells, most of which also expressed IL-2 receptor subunit CD25 and inhibitory receptor PD-1 (Fig. 2b). Therefore, we used the anti-CD3-stimulated P62 CTL clone to examine the consequences of the ligation to Nrp-1 of Sema-3A, for which recombinant (r) molecule is available, on the migratory behaviour and cytotoxic activity of these T lymphocytes in vitro.

Previous immunofluorescence experiments indicated that P62 CTL bound rSema-3A-Fc (Fig. 2c) and expressed CXCL12 receptor CXCXR4 (Supplementary Fig. 3e). Consequently, P62 T cells were able to migrate toward a gradient of rCXCL12 chemokine in vitro using two-well assays (Fig. 2d). We found that this T-cell migratory response was inhibited in the presence of rSema-3A-Fc (Fig. 2d). Similar results were obtained with TIL freshly isolated from three independent NSCLC patients showing that the interaction of human Nrp-1 with its soluble ligand Sema-3A inhibits T-cell migration toward CXCL12 gradient (Fig. 2e).

This impaired T-cell migration was correlated with inhibition by rSema-3A-Fc of the cytotoxic activity of the P62 CTL clone toward the cognate IGR-Pub target cell line (Fig. 2f) and of freshly isolated NSCLC TIL toward autologous fresh tumour cells (Fig. 2g). These results indicate that Nrp-1 triggering by its soluble ligand of the Sema-3 family Sema-3A negatively regulates migration and cytotoxicity of Nrp-1+ cytotoxic T cells, and suggest a T-cell inhibitory receptor function for Nrp-1.

Nrp-1+ CD8+ TIL in B16F10 display an exhausted PD-1hi state. To investigate the impact of Nrp-1 engagement with its ligand on CD8+ TIL functions in vivo, we used C57BL/6 mice engrafted with B16F10 melanoma cells, which express the Nrp-1 soluble ligand Sema-3B, as shown by qRT-PCR analyses (Supplementary Fig. 4a) and confirmed by western blot (Fig. 3a). At day 15, tumours were removed and TIL were isolated and analysed by flow cytometry. T cells from spleens and tumour-draining lymph nodes (TDLN) of the same mice were examined in parallel for Nrp-1 expression. Results showed that a large fraction of CD8+ TIL expressed Nrp-1 (45.5% ± 2.5), as opposed to the very low percentages in spleens (1.9% ± 0.4) and TDLN (1.3% ± 0.1). The frequency of Nrp-1+ T cells was also higher in CD4+ TIL (37.7% ± 2.6), compared with spleens (15.7% ± 0.8) and TDLN (12% ± 0.4) (Fig. 3b). CD8+ TIL isolated from engrafted lung tumour cell lines LL2 and TC-1, and the colon cancer cell line MC-38 also expressed high levels of Nrp-1, as opposed to spleens and TDLN (Supplementary Fig. 4b, c and 4d).

Although, moreover, most Nrp-1+ CD4+ cells from spleens and TDLN of B16F10-engrafted mice expressed the Treg cell marker FoxP3, Nrp-1+ CD4+ TIL included both FoxP3+ and FoxP3− T cells (Fig. 3c). It should be noted that most, if not all, CD8+ TIL displayed a CD44hiCD62Llow phenotype, characteristic of effector/memory T (TEM) cells, especially when they expressed Nrp-1 (Fig. 3d). Notably, as in humans, expression of Nrp-1 was dependent on the activation state of T cells. Indeed, stimulation of CD3+CD8+ mouse splenocytes with immobilised anti-CD3 induced Nrp-1 expression, which reached a plateau at 72 h (Supplementary Fig. 5a). It was inhibited by the INFAT inhibitor 11R-VIVIT, indicating that the TCR signalling pathway was likely involved (Supplementary Fig. 5b).

We then focused on induction of Nrp-1 on CD8+ TIL during tumour progression. Expression of well-known inhibitory receptors PD-1, CTLA-4, Tim-3 and LAG-3 was monitored in parallel. Kinetic studies revealed that the percentage of Nrp-1+ CD8+ TIL increased during tumour growth, with similar induction of PD-1, LAG-3 and Tim-3 and, to a lesser extent, CTLA-4 (Supplementary Fig. 5c). The absolute number of T cells (i.e., the number of TIL per mg of tumour) expressing these inhibitory receptors also increased during melanoma progression, and reached a peak at day 14 after tumour implantation (Supplementary Fig. 5d). Remarkably, at day 14, all Nrp-1+ CD8+ TIL also expressed PD-1, and most of which also expressed LAG-3, Tim-3 and CTLA-4 (Fig. 4a). This Nrp-1+ T-cell subset displayed a PD-1hi profile (Fig. 4a), with a strong correlation between expressions of the two cell surface molecules (Fig. 4b). Heat map analyses showed that this Nrp-1+PD-1hi TIL subset included much higher percentages of T cells expressing other T-cell inhibitory receptors than the Nrp-1−PD-1− and Nrp-1−PD-1hi subsets (Fig. 4c), with a much higher PD-1 geometric mean of fluorescence intensity (gMFI) than the Nrp-1−PD-1− subset (Fig. 4d). They also indicated that the Nrp-1+PD-1hi TIL subset included higher percentages of T cells expressing granzyme B and proliferation marker Ki-67, as well as higher gMFI of exhaustion-associated transcription factors such as NFATc1, IRF-4, Helios, Blimp-1 and T-bet, compared with the Nrp-1−PD-1− subset (Fig. 4c). Nrp-1+PD-1hi T cells also expressed high levels of Batf, Cd244 and Tigit transcripts, the products of which were associated with dysfunctional T-cell status, but not Egr2 mRNA (Supplementary Fig. 5e). It should be noted that most Nrp-1 was also found on FoxP3− CD8+ T cells expressing PD-1, and most of which also expressed PD-1, and most of which also expressed PD-1hi TIL subset included higher percentages of T cells expressing CD8+ TIL during tumour progression. Expression of well-known inhibitory Nrp-1+PD-1hi T cells. We first examined whether these lymphocytes were enriched with T cells specific to melanoma-associated antigens (MAA). To do so, C57BL/6 mice engrafted with B16F10 melanoma cells were vaccinated with Trp2 plus gp100 antigenic peptides, together with the poly(IC) adjuvant. Antigenic specificity of intra-tumoural CD8+ TIL was then analysed with H-2-Kb-Trp2 and H-2-Db-gp100 dextramers. Expression of Nrp-1 and PD-1 molecules on these TIL subpopulations was also monitored. Initial experiments showed that vaccinated mice more efficiently controlled tumour growth than unvaccinated mice (Supplementary Fig. 6a). Moreover, at day 14 after melanoma cell transplantation, tumours from vaccinated mice showed increased absolute numbers of CD8+ TIL expressing PD-1, Nrp-1 and Tim-3 and, to a lesser extent, CTLA-4 and LAG-3 (Supplementary Fig. 6b). More importantly, the percentage of Trp2-specific and gp100-specific CD8+ TIL in the Nrp-1+PD-1hi T-cell subset was over two-fold higher (8.8% ± 2.1 and 13.4% ± 1.5, respectively) than in Nrp-1−PD-1− (2.9% ± 0.6 and 6.2% ± 0.7, respectively) TIL (Fig. 5a). In contrast, the Nrp-1−PD-1− TIL subset showed very low percentages of MAA-specific T cells (0.9% ± 0.4 and 0.4% ± 0.1, respectively).

Our results also revealed that ex vivo stimulation of Nrp-1+PD-1hi TIL with autologous tumour cells induced higher percentages of IFNγ-producing T cells than in Nrp-1−PD-1− and
the Nrp-1-PD-1+ TIL subsets (Fig. 5b). Much stronger production of TNF was also found with Nrp-1-PD-1hi CD8+ TIL compared with Nrp-1-PD-1lo T cells, but in this case, percentages of TNF-producing T cells remained lower than in the Nrp-1-PD-1+ TIL population. These results could be explained by a more advanced activation stage of the Nrp-1-PD-1hi CD8+ TIL subset than the Nrp-1-PD-1+ TIL subset. We then further assessed tumour reactivity of Nrp-1-PD-1hi and Nrp-1-PD-1+ TIL by measuring CD107a expression, a marker usually used to evaluate the degranulation capacity of CTL. Our results showed that ex vivo stimulation of TIL with the cognate B16F10 cell line induced much higher percentages of CD107a+ cells among Nrp-1-PD-1hi and Nrp-1-PD-1+ TIL populations than did the Nrp-1-PD-1+ T-cell subset (Fig. 5c).

Next, we analysed the cytotoxic activity of CD8+ TIL toward autologous melanoma cells in the absence or presence of neutralising anti-Nrp-1 and/or anti-PD-1 mAb, with an isotype-matched mAb as negative control. A 4h chromium (51Cr) release assay revealed that CD8+ TIL were poorly effective in killing B16F10 tumour cells, whether blocking mAb were
Fig. 4 Expression of T-cell activation/exhaustion markers on the CD8+ TIL. a Expression of Nrp-1, PD-1, LAG-3, CTLA-4 and Tim-3 on CD8+ T cells from B16F10 TIL isolated at day 15. Right: percentages of Nrp-1 among CD8+ T cells expressing or not PD-1 (n = 26), Tim-3 (n = 19), LAG-3 (n = 19) or CTLA-4 (n = 16). b Expression of Nrp-1 on CD8+ T cells correlated with that of PD-1 (n = 38). c Expression of Nrp-1 on CD8+ TIL is restricted to a PD-1hi T-cell population. Left, Expression of Nrp-1 and PD-1 on CD8+ T cells identification of three T-cell subsets: Nrp-1+PD-1hi, Nrp-1–PD-1hi and Nrp-1–PD-1–. Right: expression of exhaustion/activation markers, functional proteins and transcription factors in Nrp-1+PD-1hi, Nrp-1–PD-1hi and Nrp-1–PD-1– CD8+ T-cell subsets. Heat maps including percentages of cells positive for expression of PD-1, Nrp-1 and PD-1 among CD8+ TIL population. Left, Expression of three molecule expression, a phenomenon in which IFNγ secreted by T cells was likely involved. Consistently, we observed such an upregulation of H2-Kb and H2-Db expression after 12 h of stimulation of tumour cells with rIFNγ, and on melanoma cells collected from in vivo tumour grafts (Supplementary Fig. 6c). Indeed, parallel experiments in which we monitored NFATc1, IRF-4, Helios, Blimp-1 and T-bet (n = 5). d gMFI of PD-1 on Nrp-1+PD-1hi, Nrp-1–PD-1hi and Nrp-1–PD-1– TIL subsets (n = 16). Results are representative of three to five independent experiments. Means ± SEM two-tailed Student’s unpaired t test a or one-way ANOVA test with Bonferroni correction. *P < 0.05; **P < 0.01; ***P < 0.001. Source data are provided as a Source Data file c.

Fig. 5 Nrp-1 blockade optimises tumour growth control by anti-PD-1. The above experiments suggested that Nrp-1 is an immune checkpoint expressed by tumour-reactive T cells that could impair their functional activities following interaction with its ligand Sema-3B. To directly test this hypothesis in vivo and therefore investigate the potential of neutralising Nrp-1 via immunotherapeutic approaches, C57BL/6 mice were engrafted with B16F10 melanoma and treated at days 6, 8, 10, 12 and 14 with anti-Nrp-1, anti-PD-1, a combination of both, or an isotype control mAb. Both tumour volume and tumour weight were followed up. Results showed that intra-tumoural (i.t.)
administration of anti-Nrp-1 mAb, or intra-peritoneal (i.p.) injection of anti-PD-1 mAb, inhibited tumour growth as compared with control treatment (Fig. 6a, b, Supplementary Table 2). In these experiments, the rational for using the intra-tumoural route to inject anti-Nrp-1 mAb was to reduce the quantity of administrated antibodies and potential side effects. Importantly, a combination of anti-Nrp-1 plus anti-PD-1 was clearly additive, with a much better control of tumour progression (Fig. 6a, Supplementary Fig. 6d). A strong reduction in tumour weight measured at the experiment endpoint was also observed (Fig.6b).

Similar experiments were conducted in C57BL/6 mice engrafted with MC-38 tumour cells and treated with anti-Nrp-1 mAb injected i.t. combined with anti-PD-1 injected i.p. A beneficial effect of anti-Nrp-1 treatment combined with anti-PD-1 could be observed (Fig. 6c, d). We checked in this model the effect of i.p. administration of anti-Nrp-1 on tumour growth and weight, and found that it was much more efficient than the i.t. route (Fig. 6c, d). Notably, when using the i.p. route of injection, anti-PD-1 plus anti-Nrp-1 combination had similar effects on tumour progression than anti-PD-1 plus anti-CTLA-4 (Supplementary Fig. 6e).

To further assess mechanisms involved in the therapeutic effect of anti-Nrp-1 plus anti-PD-1 combination, we analysed CD8+ TIL from B16F10 tumours of the different groups of mice used above. Results showed much larger CD8+ T-cell infiltrates in tumours obtained from mice treated with the anti-Nrp-1 plus anti-PD-1 combination than from mice treated with each mAb alone or the isotype control (Fig. 6e).
migrated were determined by a of H-2-Kb/-Db and PD-L1 expression on B16F10 co-cultured with CD8 and gp100 (Nrp-1, -PD-1, a combination of both mAb or an isotype control. Cells were seeded in the upper chambers of transwells and then exposed to a gradient of mice (Fig. 5f). These CD8 were also observed in this group of mice compared with control mice treated i.p. with a combination of anti-Nrp-1 and anti-PD-1 (Fig. 6j). Overall, these results demonstrate that Nrp-1 i.p. plus anti-PD-1 combination than from mice treated with anti-Nrp-1 i.t. or i.p. alone, anti-PD-1 alone or the anti-Nrp-1+/PD-1 combination in cancer immunotherapy.

Fig. 5 The Nrp-1+/PD-1+ TIL subset is enriched with activated antigen-specific CD8+ T cells. a Staining of CD8+ TIL with dextramers. C57BL/6 mice were engrafted with B16F10 and then vaccinated with Trp2 and gp100 peptides. On day 15, TIL were isolated from tumours. Right: Percentages of Trp2 (n = 8) and gp100 (n = 5) dextramer-positive T cells among Nrp-1−PD-1−, Nrp-1−PD-1+ and Nrp-1+PD-1+ CD8+ TIL. b Expression of IFNγ (n = 20) and TNF (n = 18) in Nrp-1−PD-1−, Nrp-1−PD-1+ and Nrp-1+PD-1+ CD8+ T cells. TIL were stimulated for 4 h with autologous tumour cells. c Degranulation of CD8+ TIL. TIL were stimulated with autologous tumour cells; then, T-cell subsets were analysed for expression of CD107a (n = 18). d Cytotoxicity of freshly isolated CD8+ TIL. CD8+ TIL were pre-incubated in medium or with anti-Nrp-1, anti-PD-1 or a combination of both mAb; then, cytotoxicity toward autologous tumour cells was determined. e Increase in MHC-I and PD-L1 expression on tumour cells co-cultured with autologous CD8+ TIL. Kinetic studies of H-2-Kb/-Db and PD-L1 expression on B16F10 co-cultured with CD8+ TIL. Expression profiles (left), percentages of positive cells (middle) and gMFI (right) of MHC-I (upper panels) and PD-L1 (lower panels) are shown. f Expression of perforin in CD8+ T cells. TIL were stimulated with autologous tumour cells in the absence or presence of neutralising anti-Nrp-1, anti-PD-1 or anti-Nrp-1 plus anti-PD-1 then, T-cell subsets were analysed for expression of perforin (n = 5). g Anti-Nrp-1 re-establishes migration of Nrp-1−PD-1− T cells toward B16F10. TIL were pre-incubated in medium or with neutralising anti-Nrp-1, -PD-1, a combination of both mAb or an isotype control. Cells were seeded in the upper chambers of transwells and then exposed to a gradient of B16F10 supernatant, enriched in Sema-3B, loaded in the lower chambers. The numbers of Nrp-1−PD-1−, Nrp-1−PD-1+ and Nrp-1+PD-1+ T cells that had migrated were determined by flow cytometry. Results are representative of three independent experiments. Means ± SEM one-way ANOVA test with Bonferroni correction a-g. *P < 0.05; **P < 0.01; ***P < 0.001

Multiple roles for neuropilins and semaphorins in the immune system have emerged in recent years. Sema-3A is reported to inhibit primary human T-cell proliferation and cytokine production under anti-CD3 plus anti-CD28 stimulation. It also promotes T-cell apoptosis and inhibits non-specific cytotoxic activity of NK cells in mixed lymphocyte cultures. The influence of Sema-3A on T-cell migration has also been studied. In particular, this chemorepulsive molecule has been reported to inhibit immune cell migration and response of human T cells to chemokine gradients. Chemorepulsive effects of Sema-3A on human thymocytes have been reported, as well as inhibition of T-cell migratory responses triggered by chemokine CXCL12. Accordingly, we found that soluble Sema-3A binds to Nrp-1 molecules expressed on the surface of human lung tumour-specific CTL and inhibits their migratory capacity to a gradient of CXCL12 chemokine. Importantly, our results also showed that TCR-mediated killing of autologous human lung cancer cells was inhibited in the presence of Sema-3A, highlighting a possible inhibitory role of the Nrp-1/Sema-3A axis in CTL functions.

Nrp-1 is usually not expressed by resting T cells. However, its expression is triggered after T-cell activation, suggesting that Nrp-1 is an additional T-cell activation biomarker. Regulation of Nrp-1 expression during adaptive immune responses is likely an essential element for understanding its physiological role. In this context, activated T cells derived from inflammatory environments were described as expressing this surface molecule. Indeed, Nrp-1 was highly induced on CD8+ T lymphocytes engaging self-antigen, including human melanoma TIL, supporting the hypothesis that it may correspond to a potential biomarker for dysfunctional T cells. We show here that Nrp-1 is co-expressed with CD25 and PD-1 on both CD4+ and CD8+ TIL from human NSCLC. Moreover, on CD8+ TIL, this expression is restricted to a PD-1hi T-cell subset. Various populations of CD8+ TIL have been recently described based on PD-1 expression levels: negative (PD-1−), intermediate (PD-1int) and high (PD-1hi). Interestingly, we found a very good correlation between PD-1 and Nrp-1 expression (see Fig. 4b). Thus, the aforementioned PD-1hi TIL subset likely corresponds to cells expressing the highest levels of PD-1 and Nrp-1 in our study. This is a crucial assumption, as this subset has been recently reported to correspond to an exhausted TIL subset. Accordingly, in the present study, we found that Nrp-1+PD-1hi TIL express transcription factors Helios, Blimp-1, IRF-4 and NFATc1, we show that Nrp-1 works as a negative regulator of antitumoural activities of this CTL subset, and that blocking of this receptor in vivo strongly improves tumour regression elicited by anti-PD-1 immunotherapy.

Discussion

Nrp-1 is essential in axonal guidance, through its capacity to bind class III chemoattractant receptor-like proteins, and in angiogenesis, via its interaction with VEGF and VEGF receptors. Nrp-1 is also involved in cardiovascular and neuronal development, cell migration, immunity and cancer. Indeed, tumour cells frequently express semaphorins and their receptors neuropilins and plexins, which can regulate malignant cell behaviour and contribute to malignant potential. Nrp-1 is also highly expressed on tumour vasculature, functioning as a mediator of tumour initiation and progression, associated with poor clinical outcome. For instance, high expression of Nrp-1 observed in lung cancer correlates with invasive capacity and short disease-free survival. Moreover, cancer cells often produce secreted members of the semaphorin family, including Sema-3A and Sema-3B, also contributing to tumour escape from the immune response. We now report, in both human NSCLC tumours and murine B16F10 melanoma, that Nrp-1 delineates a particular subset of CD8+ TIL, enriched with tumour antigen-specific T lymphocytes, and also expressing high levels of the PD-1 inhibitory receptor. In particular,
Co-expression of Nrp-1 and Helios on CD4⁺ T cells was also observed on TIL from human liver metastases of colorectal cancer. Another characteristic of PD-1⁺ TIL is that they display high tumour recognition capacity and are predictive of responses of human NSCLC patients to PD-1 blockade. Likewise, we found, in B16F10 tumours, that a significant percentage of Nrp-1⁺PD-1⁺ CD8⁺ TIL strongly expressed granzyme B and cell proliferation marker Ki-67, and secreted high levels of IFN-γ. Moreover, this Nrp-1⁺PD-1⁺ T-cell subpopulation was the most strongly enriched with MAA-specific CD8⁺ T lymphocytes. We therefore assume that Nrp-1 can be used as a marker for identifying this important CD8⁺ tumour-reactive TIL population. Overall, these findings suggest that, in an antitumour immune response context,
Nrp-1 is a very late T-cell activation marker and that its co-expression with high levels of PD-1 might be another characteristic of exhausted antigen-experienced CD8\(^+\) TIL.

Expression of CTLA-4, PD-1 and Tim-3 molecules was associated with T-cell exhaustion in chronic viral infections and tumour progression.\(^{46-50}\) These T-cell inhibitory receptors are important for regulating immune responses in peripheral tissues and maintaining self-tolerance. Along the same lines, Nrp-1 is induced on tolerant self-reactive CD8\(^+\) T cells expressing known regulatory receptors.\(^{16}\) In the present report, we show that Nrp-1 is often associated with a broad panel of inhibitory receptors, such as CTLA-4, Tim-3 and LAG-3, and characterizes a dysfunctional PD-1\(^{hi}\) CD8\(^+\) TIL population. This particular phenotype, characteristic of T cells infiltrating an inflamed tumour microenvironment, may further explain the paradox of tumour progression, despite the presence of an ongoing T-cell response toward malignant cells. We also show here that anti-Nrp-1 neutralising mAb, in particular when associated with anti-PD-1, re-established ex vivo the functionality of these T cells, such as increase of perforin expression and TCR-mediated cytotoxicity toward the cognate tumour. Remarkably, anti-Nrp-1 also restore the migratory capacity of Nrp-1\(^{−}\)PD-1\(^{hi}\) T cells, likely by blocking the interaction of Nrp-1 with its ligands Sema-3A or Sema-3B. Anti-PD-1 blocking mAb have no such effect. Semaphorins have been reported to govern cell migration by regulating integrin-mediated adhesion and actin cytoskeleton.\(^{51}\) A similar process may occur in tumours to explain how Nrp-1 signalling specifically affects T-cell migratory potential, possibly downstream plexin-A, which forms stable heterodimers with Nrp-1 and acts as a signal-transducing module for the Sema-3/Nrp-1/Plxn-A complex at the plasma membrane.\(^{52}\)

It is becoming increasingly clear that Nrp-1 and its ligands Sema-3A/3B play important roles during the effector phase of various immune processes, including antitumour T-cell responses. A growing body of evidence indicates that these molecules are commonly expressed by cancer cells and immune cells, and that they may affect antitumour immune responses. In this regard, it has been reported that LKB1 protein kinase\(^{53}\) and the ABL1 inhibitor Imatinib\(^{54}\) inhibit tumour growth and angiogenesis by enhancing Nrp-1 degradation. Moreover, synthetic anti-Nrp-1 peptides\(^{31}\) and blocking anti-Nrp-1 mAb have been developed\(^{55}\) for targeting tumour angiogenesis. It has also been reported that inhibition of Sema-3C binding to Nrp-1 with small molecules attenuates tumour growth in prostate cancer.\(^{56}\) In this context, our work is a building block, since we now show that Nrp-1 negatively influences CD8 T-cell immunity and responses to anti-PD-1 cancer immunotherapy. Indeed, our in vivo experiments have revealed that, like the anti-PD-1 blockade, anti-Nrp-1 immunotherapy is able to inhibit melanoma progression in C57BL/6 mice. Moreover, a combination of the two antibodies is more efficient at reducing tumour growth. In contrast, combination of anti-Nrp-1 plus anti-PD-1 did not have an additive effect on T-cell-mediated cytotoxicity ex vivo. The mechanism of the differential outcome in these two experimental systems is likely associated with the capacity of anti-Nrp-1 to enhance T-cell migration and recruitment within the tumour. Consistently, reduced tumour growth in vivo was associated with enhanced tumour infiltration by CD8\(^+\) effector T cells, with an increase in the CD8\(^{+}\)/CD4\(^{+}\) T-cell ratio. Moreover, CD8\(^+\) TIL from mice treated i.p. with anti-Nrp-1 plus anti-PD-1 expressed higher levels of granzyme B and mediated stronger cytotoxic activity toward autologous tumour cells was determined. Absolute cell counts of granzyme B\(^{+}\) CD8\(^+\) T cells from MC-38 TIL Numbers of T-cell subsets per milligram of tumour from mice treated i.p. with anti-PD-1, i.t. or i.p. with anti-Nrp-1, or a combination of anti-PD-1 (i.p.) plus anti-Nrp-1 (i.t. or i.p.) mAb. Means ± SEM two-way ANOVA test with Bonferroni correction. a, c, e or one-way ANOVA test with Bonferroni correction. b, d, f, g, j, *P < 0.05; **P < 0.01; ***P < 0.001.

Methods

Human PBL, lung tumours and freshly isolated lung TIL. Healthy donor blood samples were collected from the French blood bank (Etablissement français du Sang (EFS); agreement number N°12/EFS/079), and patient blood samples were collected from Gustave Roussy. All patients were suffering from advanced and inoperable NSCLC stage III/IV. Immune monitoring in the blood of patients was
Derivation of the P62 CTL clone and IGR-Pub tumour cell line. NSCLC cell line IGR-Pub was derived from the tumour specimen of patient Pub adenocarcinoma in one of our laboratory24. Autologous CTL clone P62 was derived from TIL of the NSCLC cell line IGR-Pub. TIL were isolated using a tumour cell isolation kit (Miltenyi 130-108-339). Autologous CTL clones were obtained from the Centre hospitalier Marie Lannelongue and the Institut mutualiste Montsouris. RNA was immediately extracted with TRIzol reagent (Invitrogen), reverse-transcribed and then subjected to qRT-PCR.

For freshly isolated TIL, human lung tumours were dissociated mechanically and enzymatically using a tumour dissociation kit (Miltenyi Biotec 130-095-929). Mononuclear cells were then isolated by Ficoll-Hypaque gradient, and epithelial tumour cells were isolated using a tumour cell isolation kit (Miltenyi 130-108-339). Human experiments were approved by the Institutional Review Board of the Gustave Roussy Institute. All recruited healthy volunteers and patients provided written informed consent.

Mouse cell lines, tumour engraftment and peptide vaccine. MC-38 colon cancer cells subcutaneously (s.c.) in the right flank of C57BL/6 J mice were inoculated for MC-38, and TIL were sorted and analysed on day 17 for B16F10 or days 4, 7, 10 and 16 after tumour inoculation for B16F10 or 20 and 28 after tumour inoculation for B16F10.

For experiments with human freshly isolated lung TIL and CTL clone P62, activated T cells were incubated for 30 min to 1 h either with BSA (Merck) or Sema-3A-Fc (R&D systems 1250-S3-025, 100 ng/ml), and their cytotoxic activity toward the human rCXCL12 (Peprotech 300-28 A, 50 nM) was evaluated. Results were expressed as chemokinesis index.

T-cell stimulation, cytokine production and cytotoxic assay. Purified CD45+ TIL were co-cultured for 4 h with B16F10 tumour cells, pulsed with Trp2 (1 µg) and gp100 (1 µg) peptides, in the presence of BrdUrd A (Invitrogen 4506-51, 1/1000), monensin (Merck M5273, 2 µM) and anti-CD107a mAb (clone 1D4B, Biolegend, 1/1000). Cytotoxic activity toward the B16F10 cell line, pulsed with Trp2 and gp100 peptides, or the MC-38 cell line was evaluated using a conventional 4 h and overnight chromium (51Cr) release assay.

For experiments with human freshly isolated lung TIL and CTL clone P62, activated T cells were incubated for 30 min to 1 h either with BSA (Merck) or Sema-3A-Fc (R&D systems 1250-S3-025, 100 ng/ml), and their cytotoxic activity toward the autologous tumour cell was evaluated.

Western blot analyses. Equivalent amounts of protein extracts from tumour cell lines were separated by SDS-PAGE and transferred to a nitrocellulose membrane61. Blots were then probed with anti-Sema-3b mAb (clone 904201, R&D systems MAC59941, 10 µg/ml), anti-PD-1 (clone RMP1-14, Bio-X-Cell BE0146, 10 µg/ml) or isotype control (clone 2A3, Bio-X-Cell BE0089, 10 µg/ml). MC-38 tumour cells were cultured for 2 days in the lower chambers of Transwell plates (Corning) and then TIL were seeded in the upper chambers to trigger T-cell migration. After 2 h at 37 °C, the number of CD8+ T cells that had migrated into the lower chambers was counted by flow cytometry and phenotyped.

For experiments with freshly isolated human lung TIL and CTL clone P62, activated T cells were incubated for 30 min to 1 h either with BSA (Merck) or Sema-3A-Fc (R&D systems 1250-S3-025, 100 ng/ml), and their cytotoxic activity toward the human rCXCL12 (Peprotech 300-28 A, 50 nM) was evaluated. Results were expressed as chemokinesis index.

In vitro migration assay. Freshly isolated CD8+ TIL were incubated for 30 min to 1 h either in medium or in the presence of neutralising anti-Nrp-1 mAb (clone 761704, R&D systems MAB59941, 10 µg/ml), anti-PD-1 (clone RMP1-14, Bio-X-Cell BE0146, 10 µg/ml) or isotype control (clone 2A3, Bio-X-Cell BE0089, 10 µg/ml). B16F10 tumour cells were cultured for 2 days in the lower chambers of Transwell plates (Corning) and then TIL were seeded in the upper chambers to trigger T-cell migration. After 2 h at 37 °C, the number of CD8+ T cells that had migrated into the lower chambers was counted by flow cytometry and phenotyped.
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Additional information

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