Characterization and modulation of CC-chemokine Receptor 6 (CCR6) mediated immunosurveillance in malignant melanoma

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Background

- Chemokines and their receptors play an essential role in the elicitation of specific immune responses, controlling compartment-specific migration of immune and tumor cells
- Chemokine ligand 20 (CCL20) expressed in the epidermis is a potent impetus for the recruitment of subsets of dendritic cells (DCs), B-cells and memory T cells expressing Chemokine receptor 6 (CCR6), its exclusive receptor
- CCL20 and a CCR6-expressing immune cell infiltrate has been detected in several malignancies, including melanoma
- The functional contribution of the CCR6/CCL20 axis in skin inflammation is well described but its role in the immune control of melanoma remains controversial

Results

1)-Characterization of CCL20 production by murine and human melanoma cell lines *in vitro*

- B16 murine melanoma cells have low homeostatic CCL20 expression but are capable to produce CCL20 upon pro-inflammatory conditions *in vitro*
- After a few passages, stimulation with pro-inflammatory cytokines cannot trigger CCL20 expression *in vitro* anymore
- Similar effects can be observed in other murine (Ret) and human (A357, C32) melanoma cell lines

2)- B16 melanoma cells with enhanced CCL20 production show slower tumor growth in CCR6ko mice

2.1) Generation of retrovirally transduced high CCL20-producer B16 cell line carrying a pLNCX2-CCL20 vector.

- B16-CCL20 cells produce CCL20 constantly under homeostatic conditions (A)
- A control cell line with weak/non existent CCL20 expression is transduced with the same empty vector (B16-Control)

B16 PMSCV cells were retrovirally transduced with pLNCX2 vector carrying a CCL20 gene (B16-CCL20) or empty (B16 Control). Cells were generated in collaboration with Prof. Rainer Will from German Cancer Research Centre (DKFZ) in Heidelberg Data in bar graphs represent means $\pm$ SD (t-test, **P<0.005, ***P<0.0005)

2.2) CCR6ko mice show slower tumor growth after s.c. Injection with B16-CCL20 cells (C)

- WT and CCR6ko mice show similar tumor growth kinetics after s.c. Injection of B16 Control cells (B)
- WT mice injected s.c. with B16-CCL20 cells show same tumor kinetics as control groups (C)

4x10⁵ cells were injected s.c. in the flank of WT (C57BL/6) and CCR6ko (C57BL/6 derived) mice. Tumor growth was measured daily using a caliper. Tumor area = (major radius)*(minor radius)* π . Data in growth curves represent means $\pm$ SEM of 25 mice in each group. Two-way ANOVA, Bonferroni post-test. (C) Statistically significant difference versus WT-CCR6ko groups, $p = 0.0008$

2.3) CCR6ko mice injected with B16-CCL20 cells start developing melanoma tumors later (D) and have lower incidence rates than the other 3 experimental groups (E)

Data taken from mice used on experiments in 2.2

Aim of the project

Evaluate the distinct functional contribution of CCR6 interactions with CCL20 in modulating specific cellular anti-melanoma immune responses

Specific aims

- ✓ Characterization of CCR6/CCL20-dependent anti-tumor immune responses in primary malignant melanoma in murine models of melanoma
- ✓ Identification of local immunomodulatory factors with functional relevance for CCR6/CCL20 mediated anti-melanoma immune responses
- ✓ Correlation of CCR6/CCL20 expression patterns in primary human melanoma and LN metastasis with respect to disease stage and local tumor progression

2.4) Local injections of CCL20 after B16-Control cell inoculation slow tumor growth in CCR6ko mice

- The results obtained using the retrovirally transduced B16-CCL20 cell line can be reproduced using the B16-Control cell line and injecting CCL20 periodically near the tumor inoculation area

4x10⁵ cells were injected s.c. in the flank of WT (C57BL/6) and CCR6ko (C57BL/6 derived) mice. Tumor growth was measured daily using a caliper. 50ng CCL20 were injected every 2 days from 2nd day after inoculation. As tumor was growing the amount of local injections was increased to 2 (when tumor $\varnothing$ > 5mm) or 3 (when tumor $\varnothing$ > 10mm). Tumor area = (major radius)*(minor radius)* π . Data growth curves represent means $\pm$ SEM of 10 mice in each group. Two-way ANOVA, Bonferroni post-test. Statistically significant difference versus WT-CCR6ko groups, $p = 0.017$

3)-¿Do melanoma tumor cells express CCR6 in response to CCL20 stimulation?

- B16 cells lines used in these experimental set-ups are not CCR6ko. Therefore an autocrine pathway may be the cause of the slower tumor growth in CCR6ko in presence of high levels of CCL20
- FACS and real-time PCR preliminary experiments have shown that B16-CCL20 cell line expresses CCR6
- The effects that the expression of CCR6 may be causing in the tumor microenvironment require further investigation

B16-CCL20 cells show an intermediate CCR6 expression when compared to the Parental and Control cell lines. 5x10⁴ Cells were cultured for 72 h before being collected for detection of CCR6 by means of FACS staining.

CCL20 and CCR6 mRNA was measured by means of qPCR in the B16 cell lines. Higher levels of CCR6 mRNA (almost double) in the B16-CCL20 cell line would correlate with CCR6 expression detected by means of FACS. 5x10⁴ Cells were cultured for 72 h before being collected. RNA was extracted and measured by means of real-time PCR. Expression fold change values are the result of comparing the $\Delta\Delta Ct$ values of both cell lines against the B16 parental cell line.

Summary and Future insights

- ✓ Human and Murine melanoma cell lines are able to produce CCL20 after stimulation with pro-inflammatory cytokines, but this capability can decrease after several passages *in vitro*. In B16 murine melanoma cells, this loss of expression can affect melanoma tumor growth when injected in CCR6ko mice
- ✓ Despite the absence of CCR6, high levels of CCL20 generate an effect that results in later tumor establishment and slower tumor growth in CCR6ko but not in WT mice when injected with retrovirally transduced B16 melanoma cells. This effect can be also observed in normal B16 melanoma cells when CCL20 is injected periodically near the tumor

inoculation site. This points out that CCL20 might be triggering systemic effects only detectable in the absence of CCR6.

- A deeper analysis of the Tumor microenvironment (Monitor CCL20 levels, identification of the immune infiltrate, characterization of CCL20 related cytokines) is being performed to elucidate the role of CCL20 and CCR6 in melanoma tumor progression
- Current experiments are focused on the possible autocrine effect of CCL20 over melanoma cells and its relation with cell proliferation and CCR6 expression.