Investigating the immune-regulatory function of *NR4A1/Nr4a1* in aggressive lymphomas

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Background

Aggressive lymphomas represent the most common type of lymphoid malignancies with a five-year survival rate of 60%. Despite effective initial treatment, one-third of all patients will experience a relapse, warranting more research to discover novel therapeutic strategies. We recently detected a significant reduction of nuclear receptor NR4A1 expression in patients with diffuse large B cell lymphomas (DLBCL) that correlated with poor cancer-specific survival.

Aim

The aim of this study is to investigate the immune-regulatory properties of *NR4A1/Nr4a1* in aggressive lymphomas.

Material & Methods

(I) **Kaplan Meier analysis** was performed for survival and tumor formation in *EμMyc Nr4a1*+/+ (n=134), *EμMyc Nr4a1*-/- (n=84) and *EμMyc Nr4a1*+/- (n=59) mice.

(II) **RNA-Seq** was conducted in a total of 10 tumors from *EμMyc Nr4a1*+/+ and *EμMyc Nr4a1* -/- mice (n=5 per group and genotype).

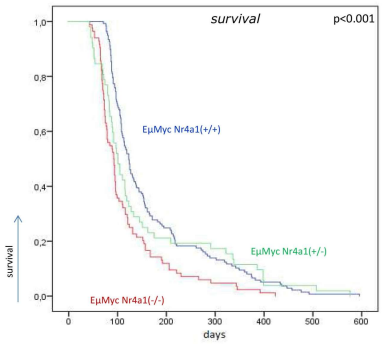
(III) Validation of the differentially expressed genes was carried out by **RQ-PCR** on fresh-frozen tumor specimens derived from *EμMyc Nr4a1*-/- and *EμMyc Nr4a1*+/+ mice (n=20 per group), including those tumors used for RNA-Seq: Moreover, the same validation was performed on tumors from mice transplanted with either *EμMyc Nr4a1*-/- (n=14) or *EμMyc Nr4a1* +/+ (n=7) derived tumors.

(IV) ***In vivo* tumor formation** was induced by injection of tumor cells derived from *EμMyc Nr4a1*+/+ and *EμMyc Nr4a1*-/- mice into the tail vein of wild type (wt) C57Bl/6 (n=20 per genotype) and Fox Chase SCID Beige (n=15 per genotype) mice.

(V) ***In vitro* cytotoxicity assay** using $OVA_{257-264}$ peptide-pulsed *EμMyc Nr4a1*+/+ and *EμMyc Nr4a1*-/- lymphoma cells and OVA targeting OT-I CD8⁺ T cells with and without *Ctla4* loss was performed to measure T cell-mediated lymphoma cell lysis.

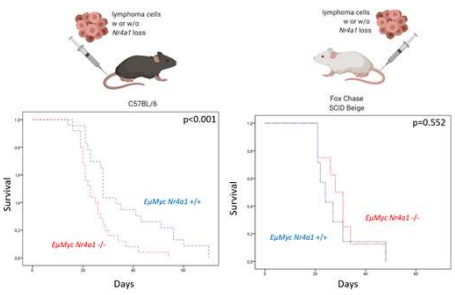
Results

Loss of *Nr4a1* augments tumor formation and decreases survival



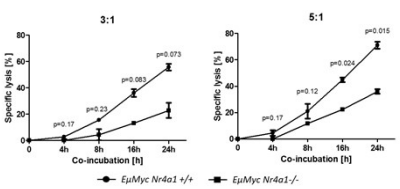
Visible tumors were developed faster in *EμMyc Nr4a1*-/- (median 45 days) compared to *EμMyc Nr4a1*+/+ mice (median 107 days, p=0.001). Moreover, *EμMyc Nr4a1*-/- showed a decreased survival with a median of 92 days compared to *EμMyc* mice without *Nr4a1* loss (median survival 123 days, p=0.037). Survival and tumor formation gave intermediate values for *EμMyc Nr4a1*+/- mice (median 101 days and 66 days, respectively).

Loss of *Nr4a1* accelerates lymphomagenesis in immunocompetent mice



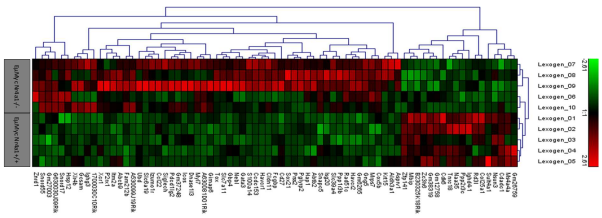
Decreased survival and a largely similar expression pattern of these immune components were found in tumors from *EμMyc Nr4a1*-/- vs. *EμMyc Nr4a1*+/+ lymphoma cells transplanted into immuno-competent C57Bl/6 mice. In contrast to immuno-competent mice, transplanting *EμMyc Nr4a1*-/- lymphomas into immuno-deficient Fox Chase SCID Beige mice exhibited an unchanged survival when compared to transplantation of *EμMyc Nr4a1*+/+ lymphomas. Interestingly, we observed an altered expression of Pd1-Pdl1-Pdl2- and Ctla4-Cd80-Cd86-axes in the immune-deficient setting. There was no expression of *Pd1* and *Ctla4* and a higher expression of *Pdl1, Pdl2, Cd80* and *Cd86* in Nr4a1 deficient lymphomas.

Reduced lymphoma cell killing in murine lymphoma cells with *Nr4a1* loss



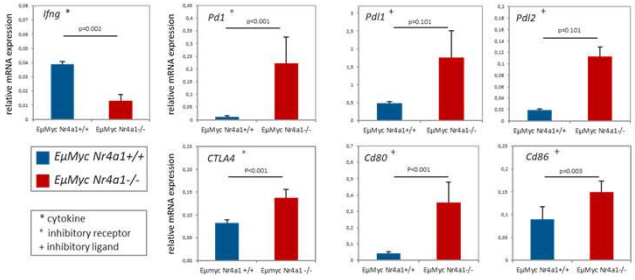
To further investigate if the *Nr4a1* expression is mandatory for active T cell-mediated anti-lymphoma immune response, we performed co-culture experiments using $OVA_{257-264}$ peptide-pulsed *EμMyc Nr4a1*+/+ and *EμMyc Nr4a1*-/- lymphoma cells and OVA targeting CD8⁺ T cells and measured T cell-mediated cell lysis. Interestingly, lymphoma cell lysis was massively diminished in *the EμMyc Nr4a1*-/- setting in ratios 3:1 and 5:1 [E:T] after 16 h and 24 h.

Genes involved in immunoregulation are upregulated upon loss of *Nr4a1*



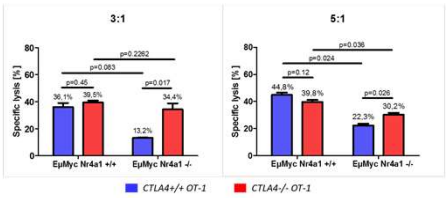
RNA-Seq was performed in a total of 10 tumors from *EμMyc Nr4a1*+/+ and *EμMyc Nr4a1* -/- mice (n=5 per genotype). By using an adjusted p-value below <0.1, 57 upregulated and 18 downregulated genes could be detected when comparing *EμMyc* mice with and without *Nr4a1* loss. GO analyses showed that mainly genes involved in immunological processes were enriched in the *EμMyc Nr4a1* -/- lymphomas.

Validation of RNA-Seq confirms influence of *Nr4a1* loss on immunomodulating molecules



In order to validate the results of the RNA-Seq, RNA was isolated from fresh-frozen tumor specimens derived from *EμMyc Nr4a1*-/- and *EμMyc Nr4a1*+/+ mice. RQ-PCR was performed on tumors from the breeding cohort including those tumors used for RNA-Seq (n=20 per genotype). Depicted results show the most important immunoregulatory molecules, which were found to be overexpressed in primary (and transplanted tumors - not shown) derived from *EμMyc Nr4a1*-/-. Moreover, expression patterns of cytokines and checkpoint components were reminiscent of our mouse data when comparing NR4A1-high and –low subjects in our DLBCL cohort. These data confirm the results obtained from our pre-clinical models.

Ctla4 loss in CD8+ T cells has no impact in lymphoma cell killing



To elucidate whether reduced lymphoma cell lysis in the *Nr4a1*-/- setting is mediated by the Ctla4-Cd80-Cd86 axis, we used *Ctla4* -/- *OT-1* CD8+ T cells instead of *Ctla4* +/+ *OT-1* CD8+ T cells in our co-culture cytotoxicity assay. Interestingly, after 16 h and 24 h of co-incubation, no significant specific lysis compared to the *Nr4a1*+/+ and *Nr4a1*-/- setting was found.

Conclusion

Our results suggest that the loss of *Nr4a1* accelerates the *Myc*-driven lymphomagenesis in immuno-competent mice. Further, these data indicate that *Nr4a1* possesses immune regulatory function and thereby contributes to the immune evasion of aggressive lymphomas by regulating T cell-mediated anti-lymphoma immune responses. Thus, it might serve as a potential target for novel immunotherapeutic approaches to treat aggressive lymphomas.