Nanobody based approaches for the therapeutic targeting of conserved T cell subsets in cancer

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Chapter 2

Circulating invariant natural killer T-cell numbers predict outcome in head and neck squamous cell carcinoma: Updated analysis with 10-year follow-up

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Head and neck squamous cell carcinoma (HNSCC) is the most common histologic tumor type of the upper aerodigestive tract. Approximately 75% of patients present with locally advanced disease and require combined modality treatment consisting of surgery and radiotherapy, or chemoradiotherapy. Tumor recurrence occurs in 60% and 5-year survival approximates 35%. Environmental carcinogens and a failure of the immune system have been implicated in the initiation and development of HNSCC.[1, 2]

Previously, we reported that a severe deficiency in invariant natural killer T (iNKT) cells, which constitute a unique CD1d-restricted immunoregulatory T-cell subset that rapidly produces large amounts of cytokines upon triggering, was related to poor outcome in HNSCC.[2] Here, we provide an updated analysis of this patient group with a median follow-up of 8.7 yr. As recent reports demonstrated human papillomavirus (HPV) status to be a strong independent prognostic factor in oropharyngeal cancer[3], we additionally evaluated tumor HPV-status in relation to iNKT-numbers in this patient subgroup.

Peripheral blood iNKT-numbers were assessed by flowcytometry in 47 HNSCC patients before the start of curative-intent radiotherapy (reference[2] details study design and patient characteristics). Patients were stratified based on iNKT-levels: low (<25th percentile), intermediate (25th-75th percentile), or high (>75th percentile). Overall survival (OS), disease-specific survival (DSS), locoregional control (LRC), and development of distant metastases (DM) were measured from start of radiotherapy until the time of first failure or the most recent follow-up if no relapse was detected. The median follow-up time was 104 (range 5-137) months. Univariate analyses demonstrated a significantly decreased OS, DSS, and LRC rate and an increased DM rate in patients with low iNKT-levels before radiotherapy (Figure 1 and Table 1). Clinical T-stage was associated with DSS and LRC but not with OS or DM; tumor differentiation grade was significantly associated with OS but not with DSS, LRC or DM. Multivariate analyses, performed using the Cox proportional hazards model, confirmed that the iNKT-level was an independent prognostic factor with regard to OS (low *v* high iNKT, HR=9.1, *P*=.002), DSS (low *v* high iNKT, HR=11, *P*=.046), and LRC (low *v* high iNKT, HR=14, *P*=.013) (Table 2). Age was a significant confounding factor with regard to OS and DSS (HR=1.1, *P*=.007 and .034 respectively). Clinical T-stage was indicative of reduced DSS (HR=5.3, *P*=.047) but not of OS. Paraffin-embedded tumor tissue from oropharyngeal cancer patients (*n*=10) was additionally tested for HPV using an initial p16 staining that when positive was followed by an HPV-PCR. Tumor biopsies from all oropharyngeal cancer patients were negative for HPV implicating that the survival benefit of patients with intermediate/high iNKT-levels was not related to HPV-infection, strengthening the hypothesis that the observed survival benefit is a direct consequence of differences in circulating iNKT. An interesting difference between OS and DSS was found in patients with intermediate iNKT-levels, as their DSS was comparable to that of patients

with high iNKT-levels (10-year DSS: 90% *v* 92%) while their OS was strikingly reduced (10-year OS: 26% *v* 70%). As a reduced size of the iNKT-pool could increase susceptibility to a secondary malignancy by hampering tumor immunosurveillance, it is interesting that an increased frequency of second primary tumors was observed in patients with intermediate iNKT-numbers (33% *v* 9%). However, univariate analyses revealed no overall statistically significant correlation between iNKT-numbers and the occurrence of a second primary tumor.

A reduced size of the circulating iNKT-pool is a characteristic of many tumor types (reviewed in [4]). Our results unequivocally demonstrate that iNKT-levels are strong predictors of clinical outcome in HNSCC patients treated with curative-intent radiotherapy. As a direct relation between a low intratumoral iNKT-frequency and poor prognosis was noted in neuroblastoma and colorectal cancer[4], the predictive value of iNKT could be a more general phenomenon. The pathogenesis of the defective iNKT-pool in cancer patients has not yet been elucidated, but might be related to the production of immunosuppressive cytokines, shedding of natural glycolipids by tumor cells, and/or by alterations in CD1d-expressing APC. [4] As the iNKT-deficiency is retained for >18 weeks after radiotherapy for HNSCC and is not affected by disease stage or surgical tumor removal[2], it appears that tumors either induce irreversible defects in the iNKT-population or that a pre-existing defect in the iNKT-population provides a risk factor for tumor development.

The present data with a median follow-up time of 8.7 years demonstrate that HNSCC patients with a severe iNKT-deficiency have a strikingly poor clinical outcome. As early iNKT-based clinical trials observed anti-tumor activity in HNSCC[5], we believe sufficient rationale exists to further explore iNKT-based immunotherapeutic strategies in HNSCC.

Table 1. Log-Rank Statistics

	Primary Tumor*								Secondary Unrelated Tumor†	
	OS		DSS		LRC		DM		TFS	
Clinical Parameter	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*
Sex	1.219	.270	0.432	.511	1.209	.271	2.096	.148	0.007	.935
Primary *v* postoperative RTH	0.030	.853	1.416	.234	0.072	.788	0.046	.830	3.394	.065
T1-T2 *v* T3-T4‡	3.538	.060	6.064	.014§	6.353	.012§	0.745	.388	1.043	.307
N0 *v* N+	0.000	.993	1.766	.184	0.005	.945	2.361	.124	0.888	.346
Grades 1-2 *v* 3-4¶	3.892	.049§	0.003	.958	0.274	.601	0.301	.583	0.884	.347
iNKT cells/10^6 T cells	8.43	.015§	9.689	.008§	11.730	.003§	8.331	.016§	1.689	.430
iNKT cells/mL	22.77	.000§	8.024	.018§	13.528	.001§	12.967	.002§	1.244	.537
T cells/mL	5.99	.051	0.616	.735	2.873	.238	3.666	.160	0.586	.746
NK cells/mL	4.08	.130	2.350	.309	0.590	.745	1.028	.598	1.697	.428

NOTE: Patients were stratified before the start of radiotherapy on the basis of sex, treatment, disease stage, tumor grade, or iNKT-, T-, or NK-cell levels (< the 25th percentile, 25th to 75th percentile, and > the 75th percentiles of the total population). *P* values were determined by log-rank statistics. Significant associations were further investigated in a multivariate analysis (Cox regression model).

Abbreviations: DM, development of distant metastases; DSS, disease-specific survival; iNKT, invariant natural killer T; LRC, locoregional control; N+, lymph node metastases detected; N0, absence of lymph node metastases; NK, natural killer; OS, overall survival; RTH: radiotherapy; TFS, tumor-free survival.

*Relative differences in primary tumor–related OS, DSS, LRC, and DM event distribution between stratified groups (χ^2).

†Relative differences regarding TFS of a secondary unrelated tumor.

‡Grades 1-2 denote a well-differentiated to moderately differentiated tumor, and grades 3-4 denote a poorly differentiated to undifferentiated tumor.

§$P < .05$

¶T1-T4 denote the extent of the primary tumor according to the TNM classification (International Union Against Cancer criteria of tumor response 1997).

Table 2. Cox Regression

Parameter	*P*	Relative Risk	95% CI
OS*	.001		
iNKT/10^6 T cells	.005		
< 48 *v* 48-242	.019	3.0	1.2 to 7.4
< 48 *v* > 242	.002	9.1	2.2 to 37
Age	.007	1.1	1.0 to 1.1
Grades 1-2 *v* 3-4†	.054	NA‡	NA‡
DSS*	.003		
iNKT/10^6 T cells	.030		
< 48 *v* 48-242	.017	11	1.5 to 71
< 48 *v* > 242	.046	11	1.0 to 125
Age	.034	1.1	1.0 to 1.2
T1-T2 *v* T3-T4§	.047	5.3	1.0 to 28
LRC*	.003		
iNKT/10^6 T cells	.012		
< 48 *v* 48-242	.027	3.2	1.1 to 9.0
< 48 *v* > 242	.013	14	1.7 to 111
Age	.215	NA‡	NA‡
T1-T2 *v* T3-T4§	.064	NA‡	NA‡
DM*	.016		
iNKT/10^6 T cells	.428	NA‡	NA‡
< 48 *v* 48-242	.963	NA‡	NA‡
< 48 *v* > 242	.193	NA‡	NA‡
Age	.268	NA‡	NA‡

NOTE: Patients were stratified in three groups before to the start of radiotherapy as follows: patients with iNKT/10^6 T cells below the 25th percentile (< 48), within the 25th and 75th percentiles (48-242) and above the 75th percentile (> 242). The stepwise forward Cox proportional hazard model was used to investigate the predictive value of the iNKT level and age with regard to OS, DSS, LRC, and DM.

Abbreviations: DM, development of distant metastases; DSS, disease-specific survival; iNKT, invariant natural killer T; LRC, locoregional control; NA, not applicable.

**P* values refer to the overall significance of the regression model (omnibus test).

†Grades 1-2 denote a well-differentiated to moderately differentiated tumor, and grades 3-4 denote a poorly differentiated to undifferentiated tumor.

‡Parameter was not significantly associated with relative risk for the event (*P* > .05).

§T1-T4 denote the extent of the primary tumor according to the TNM classification (International Union Against Cancer criteria of tumor response 1997).

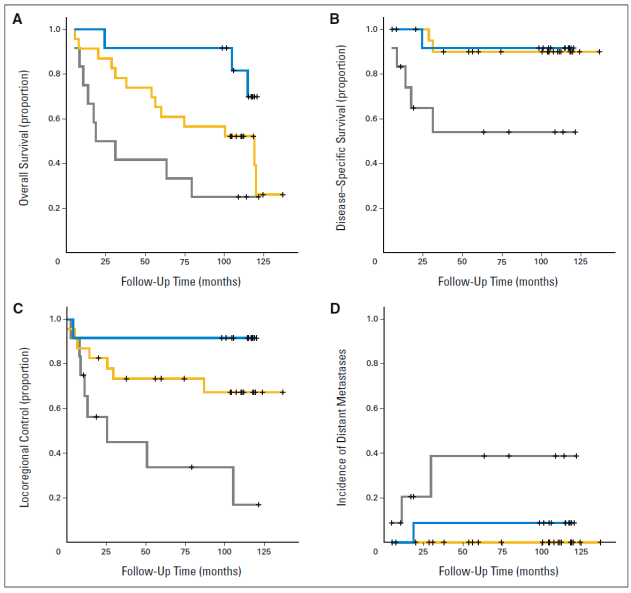

Figure 1. Kaplan-Meier analyses of OS, DSS, LRC, and DM in patients categorized according to the amount of iNKT/10^6 T cells. Patients were stratified in three groups prior to the start of radiotherapy: patients with iNKT cells / 10^6 T cells below the 25th percentile (<48, n=12, dashed line), within the 25th – 75th percentile (48-242, n=23, solid line) and above the 75th percentile (>242, n=12, bold line). Rates for iNKT low, intermediate, and high iNKT groups: (A) 10-year OS rates: 25, 26 and 70%; (B) 10-year DSS rates: 54, 90 and 92%; (C) 10-year LRC rates: 17, 67 and 92%; (D) 10-year DM-free rates: 54, 100, and 92%.

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