SNP array,[4] in total 80 cases (Supplementary Table 1). Only patients that had been treated within the Nordic Society for Pediatric Hematology and Oncology ALL 1992/2000 protocols were included in the survival analyses, comprising 48 cases including cases 2–4, 7 and 9. The probabilities of 5 years event-free survival (pEFS) and overall survival (pOS) were calculated using the Kaplan–Meier method; groups were compared with the log rank test. There were no differences in gender distribution ($P = 0.343$; two-tailed Fisher exact probability test), white blood cell count ($P = 0.803$; two-sided Mann–Whitney test), or pEFS (partial 17q gains: 0.60, SE 0.22; no partial 17q gains: 0.78, SE 0.07; $P = 0.292$) but patients with partial 17q gain were significantly older, with a median age of 6.5 years (range 3–13 years) at diagnosis compared with 3 years (range 1–16 years) for those without partial 17q gain ($P = 0.0128$; two-sided Mann–Whitney test), and had a lower pOS (partial 17q gains: 0.60, SE 0.22; no partial 17q gains: 0.93, SE 0.04; $P = 0.0200$). Although based on a small number of cases, this suggests that partial 17q gain is a marker for poor prognosis, in line with some previous reports—albeit debated—of a poor outcome in i(17q)-positive high hyperdiploid childhood ALL.[2,3,12] To ascertain whether partial 17q gains may be more common in relapsing high hyperdiploid ALL, we investigated published series of SNP array data, finding that 1/6 (17%) cases reported by Mullighan *et al.*[13] had partial 17q gain at diagnosis versus 2/6 (33%) at relapse, and 6/16 (38%) versus 8/16 (50%) cases reported by Inthal *et al.*[14] On the other hand, none of the 11 relapse cases investigated by us displayed partial 17q gains.[15] Taken together, however, there may be an increased frequency of partial 17q gain in relapsing high hyperdiploid ALL already at the time of diagnosis, becoming even more common at relapse.

In summary, we have shown that partial gain of 17q, arising through different types of chromosomal rearrangements, is a recurrent aberration in high hyperdiploid childhood ALL. The most likely pathogenetic outcome is dosage effects of genes on 17q, possibly in addition to an as yet unidentified mutation in 17p. Furthermore, the aberration may be associated with a decreased pOS and relapsing ALL.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Molecular mutations are prognostically relevant in AML with intermediate risk cytogenetics and aberrant karyotype

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The karyotype[1] and molecular mutations are well-established prognostic parameters in patients with acute myeloid leukemia (AML). Molecular mutations were identified in the vast majority of patients with normal karyotype AML (NK-AML). Mutations of the nucleophosmin (*NPM1*) gene[2] are prognostically favorable when they occur as isolated mutations in NK-AML. *FLT3*-ITD (internal tandem duplications within the *FLT3* gene) confer an adverse prognosis,[3] even in case of coincidence with the *NPM1* mutations.[4–7] Also in patients receiving allogeneic hematopoietic stem cell transplantation, occurrence of *FLT3*-ITD adversely affects relapse incidence and leukemia-free survival.[8] Alterations of the *CEBPA* gene mediate an independent favorable prognostic impact in NK-AML (at least when they occur as double mutations).[9,10] Partial

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tandem duplications within the *MLL* gene (*MLL*-PTD) are prognostically unfavorable in NK-AML.[11] The prognostic impact of these molecular mutations was mainly explored in the subset of NK-AML,[12] but only few studies evaluated the impact on prognosis in AML patients with aberrant and prognostically intermediate karyotypes. In a previous study focusing on the *NPM1* mutations, we found no difference in survival among *NPM1*-mutated AML patients between normal or aberrant karyotype (AK) cases.[13] However, so far the incidence and prognostic impact of these molecular mutations in AML with AKs had not been determined in a comprehensive study.

To clarify in which subset of AML patients a mutation screening for the respective molecular markers is reasonable, we investigated a large cohort of 1981 patients at diagnosis of AML. We aimed to determine the frequency of the *NPM1*, *CEBPA*, *FLT3*-ITD and *MLL*-PTD mutations in the different cytogenetic risk groups. Further, we evaluated the prognostic impact in the intermediate risk cytogenetic category separating AK from the NK cases to determine whether or not these mutations also impact on prognosis in cases with AKs comparable to those with NK. Bone marrow and/or peripheral blood samples were sent to the MLL Munich Leukemia Laboratory in the period from August 2005 to December 2011. Basis for the inclusion of patients in this retrospective study was the availability of the karyotype and the respective molecular markers in parallel. There were 1075 males and 906 females (median age, 68 years; range, 1–100 years; 1970 adult patients $\geqslant$18 years; 11 pediatric patients). A total of 1604 patients (81.0%) had *de novo* AML, 208 (10.5%) had secondary AML following myelodysplastic syndromes (s-AML) and 169 (8.5%) had therapy-related AML (t-AML). All patients gave their written informed consent with genetic analysis and scientific studies. The study was performed in accordance with the Helsinki Declaration. Chromosome banding analysis (combined with fluorescence *in situ* hybridization, FISH, when necessary) was performed in all cases. Patients were analyzed for *NPM1* ($n=1646$),[4] *FLT3*-ITD ($n=1726$),[3] *CEBPA* ($n=1324$)[14] and *MLL*-PTD ($n=1656$) mutations. Overall survival (OS) was calculated according to Kaplan–Meier method and compared by log-rank test. SPSS version 19.0 (IBM Corporation, Armonk, NY, USA) was used for statistical analysis.

According to the revised Medical Research Council (MRC) criteria,[1] 170 cases (8.6%) were assigned to the favorable, 1414 (71.4%) to the intermediate and 397 (20.0%) to the unfavorable MRC risk category. In the total cohort, the frequency of the mutations was as follows: most frequent were *NPM1* mutations ($n=521/1646$; 31.7%), being followed by *FLT3*-ITD: 393/1726 (22.8%). *CEBPA* mutations were detected in 102/1324 (7.7%) cases. *CEBPA* showed double mutations in 52 cases, monoallelic in 40 cases and homozygous mutations in 10 cases. Data from our group shows that homozygous *CEBPA* mutations differ in various aspects from cases with two *CEBPA* mutations (*CEBPA* double mutated)[15] and thus were separated from the latter. *MLL*-PTD were diagnosed in 123/1656 (7.4%) of all cases investigated.

The frequency of all molecular markers investigated was considerably higher in the intermediate MRC risk group (*NPM1* mutations: 511/1245; 41.0%; *CEBPA* mutations: 99/1120; 8.8%; *FLT3*-ITD: 346/1253; 27.6%; *MLL*-PTD: 106/1247; 8.5%) as compared with the favorable or unfavorable MRC risk groups (Table 1a′). Within the intermediate MRC category, the frequency of the

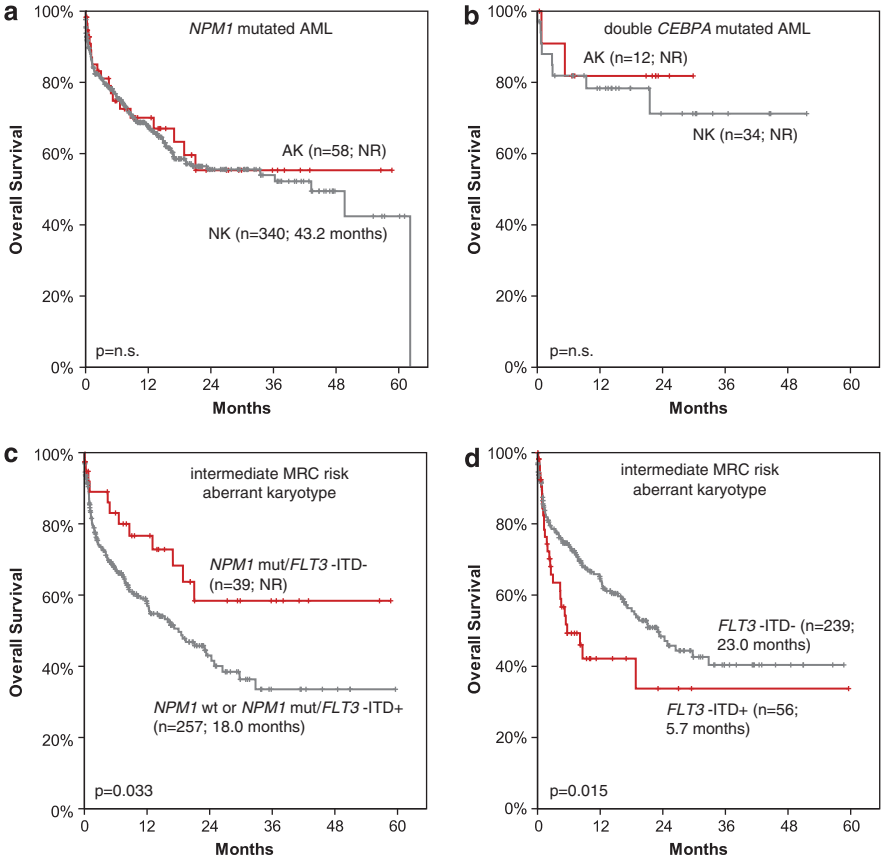

Figure 1. (**a**, **b**) OS for intermediate MRC risk patients with NK (gray) versus aberrant karyotypes (AK; red) in (**a**) *NPM1*-mutated and (**b**) double *CEBPA*-mutated AML cases. (**c**, **d**) OS for intermediate MRC risk patients with AK separated in (**c**) *NPM1*-mutated/*FLT3*-ITD-negative (*NPM1*mut/*FLT3*-ITD-; red) and *NPM1*-wild-type (*NPM1*wt) or *NPM1*mutated/*FLT3*-ITD-positive (*NPM1*mut/*FLT3*-ITD+; gray) cases and (**d**) *FLT3*-ITD-positive (*FLT3*-ITD+; red) and *FLT3*-ITD-negative cases (*FLT3*-ITD−; gray). Numbers of patients with available follow-up data and median OS are given in brackets. NR, median OS not reached.

mutations was higher in NKs as compared with the AKs of the respective category. However, the mutations were more frequent in several distinct cytogenetic subsets (for example, +4, +8, +11, +13, +21) as compared with other cytogenetic subgroups from the intermediate MRC category. *NPM1* mutations, *CEBPA* mutations and *MLL*-PTD were not observed in the reciprocal rearrangements of the favorable MRC category, in 11q23/*MLL*- or 3q26/*EVI1*-rearranged AML. The only exception were the *FLT3*-ITD, which were found in 21/68 (30.9%) of all t(15;17)/*PML-RARA* rearranged cases and in 4/6 (66.7%) cases with t(6;9)/*DEK-NUP214*. In AML with complex karyotype, all four molecular markers showed a frequency below 5% (Table 1a).

Prognosis was evaluated within the subgroup of intermediate karyotypes only, with follow-up data being available in 1028 (709 NKs, 319 AKs) out of the 1414 cases. The median follow-up was 15.4 months. In the intermediate MRC risk group, OS of patients with NKs was compared with patients with aberrant intermediate karyotypes within each molecular mutation subgroup. Within the subgroup of *FLT3*-ITD-mutated cases, patients with NK showed a non-significant trend to a longer OS as compared with AKs (median not reached vs 21.5 months; $P = 0.078$). Survival did not differ significantly between normal and AKs within *NPM1* mutated (Figure 1a), *CEBPA* monoallelic (data not shown) or double mutated patients (Figure 1b), or patients with *MLL*-PTD (data not shown).

Subsequently, we analyzed the prognostic impact of the respective molecular mutations within all patients from the intermediate MRC category (thus combining normal and AKs). OS was significantly longer in patients with *NPM1*-mutated (median 49.6 vs 18.6 months; $P = 0.003$) or *CEBPA*-mutated patients (median not reached vs mOS 21.1 months; $P = 0.016$) compared with the respective group without these mutations. Separate analysis of double and monoallelic *CEBPA* mutations revealed that only double *CEBPA* mutations were associated with a longer OS ($P = 0.006$) as compared with monoallelic/wild-type cases, whereas cases with monoallelic mutations did not significantly differ in OS from cases with *CEBPA*-wild type. OS of patients with *FLT3*-ITD was significantly shorter as compared with *FLT3*-ITD-negative patients (median 13.8 vs 24.9 months; $P = 0.003$), and *MLL*-PTD-positive patients had shorter OS as compared with *MLL*-PTD-negative patients (median 10.8 vs 23.0 months; $P = 0.039$).

Restricting the analysis to patients with AKs within the intermediate MRC category *NPM1*-mutated patients revealed a trend to a longer OS compared with *NPM1* wild type (median not reached vs 18 months; $P = 0.078$). *NPM1*-mutated/*FLT3*-ITD-negative patients revealed a significantly longer OS as compared with all other combinations of *NPM1* mutations and *FLT3*-ITD (median not reached vs 18.0 months; $P = 0.033$; Figure 1c), and a shorter OS for patients with *FLT3*-ITD as compared with *FLT3*-ITD-negative patients (median 5.7 vs 23.0 months; $P = 0.015$; Figure 1d). These results in AKs of the intermediate MRC category were corresponding to our previously published data in NK-AML, showing a clear survival benefit for the *NPM1*-mutated/*FLT3*-ITD-negative patients, whereas the survival of patients with an *NPM1*-mutated/*FLT3*-ITD-positive status did not differ significantly from the *NPM1*-wild-type/*FLT3*-ITD-positive patients.[4]

Furthermore, in our cohort of AKs from the intermediate MRC category, a positive *CEBPA* mutation status conferred longer OS as compared with *CEBPA*-wild-type patients (median not reached vs 18.0 months; $P = 0.022$). In detail, a trend to longer OS was observed for double mutations ($P = 0.062$) as compared with monoallelic/wild-type cases, but not for monoallelic *CEBPA* mutations (the 10 homozygously *CEBPA*-mutated cases were excluded from these calculations due to the limited size of this subgroup). These results were in accordance with the previous data from the literature on AML with NK, demonstrating that only double *CEBPA* mutations[9] mediate an independent favorable prognostic effect.[10] In the present study, the *MLL*-PTD were only associated with shorter OS in the total cohort with intermediate risk karyotype and in the subset with NK, whereas an impact on OS in patients with AKs was not observed (Table 1b).

In conclusion, *MLL*-PTD, *FLT3*-ITD and mutations in *NPM1* and *CEBPA* are rare in the unfavorable cytogenetic subset. In the favorable cytogenetic category only *FLT3*-ITD are observed at a mentionable frequency in patients with t(15;17)/*PML-RARA*. Further, the *NPM1* mutations, *FLT3*-ITD and *CEBPA* mutations were proven here for the first time to be prognostically relevant in patients with AKs from the

Table 1a. Frequency of molecular mutations in different MRC risk groups[1] and in distinct cytogenetic subgroups

Cytogenetic parameter	NPM1*mut (%)*	CEBPA*mut (%)*	FLT3-*ITD (%)*	MLL-PTD *(%)*
MRC risk group				
Favorable risk ($n = 170$)	0/87 (0.0)	0/19 (0.0)	26/156 (16.7)	0/91 (0.0)
Intermediate risk ($n = 1414$)	511/1245 (41.0)	99/1120 (8.8)	346/1253 (27.6)	106/1247 (8.5)
Unfavorable risk ($n = 397$)	10/314 (3.2)	3/185 (1.6)	21/317 (6.6)	17/318 (5.3)
Total ($n = 1981$)	521/1646 (31.7)	102/1324 (7.7)	393/1726 (22.8)	123/1656 (7.4)
Cytogenetic subgroups				
t(15;17)/*PML-RARA*	0/4 (0.0)	0/14 (0.0)	21/68 (30.9)	0/5 (0.0)
t(8;21)/*RUNX1-RUNX1T1*	0/42 (0.0)	0/3 (0.0)	2/42 (4.8)	0/43 (0.0)
inv(16)/t(16;16)/*CBFB-MYH11*	0/41 (0.0)	0/2 (0.0)	3/46 (6.5)	0/43 (0.0)
Normal karyotype	438/857 (51.1)	72/789 (9.1)	274/864 (31.7)	80/858 (9.3)
11q23/*MLL* rearrangements	0/57 (0.0)	0/16 (0.0)	2/58 (3.4)	0/58 (0.0)
t(6;9)/*DEK-NUP214*	0/4 (0.0)	0/5 (0.0)	4/6 (66.7)	0/4 (0.0)
3q26/*EVI1* rearrangements	0/18 (0.0)	0/10 (0.0)	0/18 (0.0)	0/18 (0.0)
+4	9/16 (56.3)	0/14 (0.0)	5/15 (33.3)	0/14 (0.0)
+8	16/82 (19.5)	4/70 (5.7)	16/83 (19.3)	6/82 (7.3)
+11	0/22 (0.0)	1/21 (4.8)	6/22 (27.3)	10/22 (45.5)
+13	1/23 (4.3)	3/24 (12.5)	5/23 (21.7)	3/23 (13.0)
+21	4/17 (23.5)	6/18 (33.3)	3/17 (17.6)	1/17 (5.9)
del(5q)	0/22 (0.0)	0/21 (0.0)	3/23 (13.0)	5/23 (21.7)
del(7q)/−7	0/26 (0.0)	2/28 (7.1)	0/26 (0.0)	0/26 (0.0)
Complex karyotypes	7/189 (3.7)	1/81 (1.2)	8/190 (4.2)	8/191 (4.2)
Other abnormalities	46/226 (20.4)	13/208 (6.3)	41/225 (18.2)	10/227 (4.4)

Abbreviations: del, deletion; *FLT3*-ITD, internal tandem duplications within the *FLT3* gene; *MLL*-PTD, partial tandem duplications within the *MLL* gene; MRC, Medical Research Council; mut, mutation; *NPM1*, nucleophosmin.

Table 1b. mOS depending on molecular markers in the MRC[1] intermediate risk category, focusing first on all patients, and subsequently on those with normal karyotypes and aberrant karyotypes, respectively

Molecular marker (no. of cases analyzed)	*Status*[a]	*Intermediate MRC category*								
		All patients (n = *1028*)			*Normal karyotype* (n = *709*)			*Aberrant karyotypes* (n = *319*)		
		No.	*mOS (months)*	P-*value*	*No.*	*mOS (months)*	P-*value*	*No.*	*mOS (months)*	P-*value*
NPM1 mutation ($n = 956$)	1	398	49.6	0.003	340	43.2	0.036	58	NR	0.078
	0	558	18.0		320	19.5		238	18.0	
FLT3-ITD ($n = 961$)	1	261	13.8	0.003	205	15.9	0.023	56	5.7	0.015
	0	700	24.9		461	33.5		239	23.0	
*NPM1*mut/*FLT3*-ITD − ($n = 957$)	1	238	49.6	< 0.001	199	49.6	0.002	39	NR	0.033
	0	719	18.0		462	19.2		257	18.0	
CEBPA mutation ($n = 874$)	1	89	NR	0.016	66	NR	NS	23	NR	0.022
	0	785	21.1		551	30.0		234	18.0	
Double *CEBPA* mutation ($n = 864$)[b]	1	46	NR	0.006	34	NR	0.044	12	NR	0.062
	0	818	21.5		577	30.0		241	18.8	
Monoallelic *CEBPA* mutation ($n = 864$)[b]	1	34	23.3	NS	26	20.5	NS	8	23.3	NS
	0	830	24.3		585	33.5		245	18.9	
MLL-PTD ($n = 957$)	1	76	10.8	0.039	58	10.1	0.028	18	18.8	NS
	0	881	23.0		604	30.0		277	19.4	

Abbreviations: *FLT3*-ITD, internal tandem duplications within the *FLT3* gene; *MLL*-PTD, partial tandem duplications within the *MLL* gene; mOS, median overall survival; MRC, Medical Research Council; mut: mutation; *NPM1*, nucleophosmin; *NPM1*mut/*FLT3*-ITD −, *NPM1*-mutated, *FLT3*-ITD-negative. [a]Mutation status: 1 positive; 0 negative. [b]Homozygous *CEBPA*-mutated cases ($n = 10$) were excluded from this analysis.

intermediate MRC risk category. This suggests to extend molecular screening for *NPM1*, *FLT3*-ITD, *CEBPA* and *MLL*-PTD mutations to all AML patients with intermediate risk cytogenetics, and not to limit analysis for these mutations to the subgroup of patients with a NK only. This will allow a better characterization of a reasonable number of cases from the intermediate risk cytogenetic category and may pave the way to more detailed risk stratification at diagnosis and therefore improved therapeutic strategies in patients with this specific subgroup of AML.

CONFLICT OF INTEREST

TH, WK, SS and CH declare part ownership of the MLL Munich Leukemia Laboratory GmbH. UB and TA work for the MLL Munich Leukemia Laboratory GmbH.

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AUTHOR CONTRIBUTIONS

CH and SS performed the study design. UB, TA, WK and CH performed data analysis. UB wrote the first manuscript draft. Classification of the cases was performed by TH (cytomorphology) and WK (immunophenotyping). SS was responsible for molecular analyses, CH for cytogenetic studies. Figures were designed by TA. All the authors contributed to the writing of the manuscript, and reviewed and approved the final version.

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Encapsulated mesenchymal stem cells for *in vivo* immunomodulation

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Mesenchymal stem cells (MSCs) are self-renewable, multipotent progenitor cells able to differentiate into various mesodermal lineages.[1] MSCs are present in basically all tissues, and have a pivotal role in tissue repair and in local control of inflammation.

In vitro studies indicated that MSCs have immunomodulatory activities: they inhibit T and NK cell activation, B-cell terminal differentiation and dendritic cell maturation and functions.[2] Little is known about the action of MSCs *in vivo* but a recent report showed that MSCs affect dendritic cell homing to lymph nodes, thus impairing T-cell priming.[3] Although there is evidence from *in vivo* studies that MSCs are able to reduce inflammatory damage without engraftment,[4] it is not clear whether their immunomodulatory effects rely on soluble mediators or cell–cell contacts (reviewed in Yagi *et al.*[2]).

There is a growing interest in using MSCs to treat human inflammatory diseases, including severe (grade III–IV) steroid resistant acute graft versus host disease (aGVHD). Various trials reported non-homogeneous results, with MSCs responses varying from 15 to 55% of treated patients. Although a study using a large cohort of patients provided encouraging results (reviewed in Tolar *et al.*[5]), recent data could not confirm the efficacy of MSCs for the treatment of GVHD.[6] The reasons for these conflicting results are not clear and, among others, may include differences in the number of MSCs that remain viable in patients overtime, a critical factor that so far is very difficult to control.

Another critical aspect of MSC-based cell therapy is its safety, especially when considering long-term complications: MSCs may cause tumor formation[7,8] or aberrantly differentiate after ectopic engraftment.[9] Finally, another risk factor is associated with the administration route. Indeed, although the number of MSCs required to achieve immunomodulation *in vivo* is basically unknown, injection of large number of cells may be necessary to obtain maximal clinical benefit given the low rate of cell retention and survival. In these conditions, MSCs may form aggregates that could cause pulmonary emboli or infarctions in patients.[10]

Considering all these issues, we decided to study the immunomodulatory effects of encapsulated mouse MSCs (E-MSCs). Indeed, this procedure would allow not only to solve all safety problems related to MSCs administration, but also to design precise protocols in which cell viability, and therefore cell dosage, is standardized. Importantly, this approach might provide conclusive data in support of either the endocrine or the paracrine/contact hypothesis for the *in vivo* immunomodulatory activity of MSCs.

Alginate is the most commonly employed polymer for cell microencapsulation because of its excellent biocompatibility and *in vivo* stability.[11] MSCs derived from B6 mice were used for the preparation of alginate microcapsules that, when analyzed by optical microscopy, showed uniform and spherical morphology (Figure 1a), with an average diameter of 600–700 μm. Staining with fluorescein diacetate and ethidium bromide showed that the microencapsulation procedure did not affect MSCs viability, even after 7 days of *in vitro* incubation (Figure 1b). In order to demonstrate that E-MSCs do not lose their multipotentiality, we compared the osteogenic and adipogenic differentiation of MSCs extracted from 7-day-old microcapsules with that of control MSCs. Both populations displayed analogous positivity for Alizarin Red staining after 15 days of conditioning with osteogenic stimuli (Supplementary Figure 1a). Similarly, the staining with Oil Red after adipogenic stimulation showed comparable differentiation

Figure 1. OVA-specific T-cell proliferation is hampered by E-MSCs. (**a**) Phase-contrast microscopy of mouse MSC-loaded microcapsules shows their uniform size and cell distribution (average capsule diameter 0.6 mm; scale bar 200 μm). (**b**) Fluorescence images of fluorescein diacetate viability assay of: MSCs monolayer before the encapsulation procedure (−) (scale bar 30 μm) and after encapsulation at day 0 and 7 in culture (scales bar 200 μm). Red staining indicates dead cells. (**c**) Diagram of the experimental protocol designed to investigate the influence of MSCs upon the activation of OT2 (OVA-specific) T cells: OT2 CD45.1 CFSE-labeled cells (1×10^6) were transferred into wild-type mice. After 24 h mice were immunized with 100 ng $OVA_{323–339}$ peptide in complete Freund's adjuvant by s.c. injection into the footpad. On day 2 a group of animals received 0.4×10^6 MSCs intravenously and another group received 0.4×10^6 encapsulated MSCs injected s.c. in the back. Four days after immunization, the popliteal dLNs were collected, counted (**d**), stained for CD4, CD8 and CD11c and analyzed by flow cytometry to determine absolute numbers (**e, f, g**). The same cell suspensions were stained to identify the $CD4^+$ $CD45.1^+$ population (**h**), with one representative dot plot shown in (**i**). $CD4^+CD45.1^+$ cells were analyzed for CFSE expression and data are expressed as percentage of cells that have divided (**j**). One representative histogram is shown in (**k**). The percentage of $CD4^+FoxP3^+$ cells was determined by i.c. staining with FoxP3 antibodies (**l**). The results of 1 representative experiment out of 3 are shown ($n = 3$ or 4; * $P<0.05$; ** $P<0.005$). CFSE, carboxyfluorescein succinimidyl ester; NS, not significant; PI, propidium iodide.

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