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$Rag2^{-/-};\gamma c^{-/-}$ immunodeficient mice, a new preclinical model to study antitumor approaches

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ABSTRACT

Animal models have been relevant to study the molecular mechanisms of cancer and to develop new antitumor agents. Anyway, the huge divergence in mouse and human evolution made difficult the translation of the gained achievements in preclinical mouse based studies. The generation of clinically relevant murine models requires their humanization both concerning the creation of transgenic models and the generation of humanized mice in which to engraft a functional human immune system, and reproduce the physiological effects and molecular mechanisms of growth and metastasization of human tumors. In particular, the availability of genotypically stable immunodepressed mice able to accept tumor injection and allow human tumor growth and metastasization would be important to develop anti-tumor and anti-metastatic strategies. Recently, $Rag2^{-/-};\gamma c^{-/-}$ mice, double knockout for genes involved in lymphocyte differentiation, had been developed (CIEA, Central Institute for Experimental Animals, Kawasaki, Japan). Studies of human sarcoma metastasization in $Rag2^{-/-};\gamma c^{-/-}$ mice (lacking B, T and NK functionality) revealed their high metastatic efficiency and allowed the expression of human metastatic phenotypes not detectable in the conventionally used *nude* murine model. *In vitro* analysis to investigate the molecular mechanisms involved in the specific pattern of human sarcomas metastasization revealed the importance of liver-produced growth and motility factors, in particular the insulin-like growth factors (IGFs). The involvement of this growth factor was then demonstrated *in vivo* through inhibition of IGF signalling pathway. Due to the high growth and metastatic propensity of tumor cells, $Rag2^{-/-};\gamma c^{-/-}$ mice were used as model to investigate the metastatic behavior of rhabdomyosarcoma cells engineered to improve the differentiation.

It has been recently shown that this immunodeficient model can be reconstituted with a human immune system through the injection of human cord blood progenitor cells. The work illustrated in this thesis revealed that the injection of different human progenitor cells ($CD34^+$ or $CD133^+$) showed peculiar engraftment and differentiation abilities. Experiments of cell vaccination were performed to investigate the functionality of the engrafted human immune system and the induction of specific human immune responses. Results from such experiments will allow to collect informations about human immune responses activated during cell vaccination and to define the best reconstitution and experimental conditions to create a humanized model in which to study, in a preclinical setting, immunological antitumor strategies.

PRECLINICAL MURINE MODELS IN BIOMEDICAL RESEARCH

In the last decades progress in biomedical research took advantage from the availability of several animal models in which to study complex biological processes with a particular attention to human cell and tissue physiology and differentiation, tumor growth and metastatic ability. Although mice represent the most used experimental mammalian model system, they are separated from humans by million years of evolutions. Several species differences in the structure and function of the immune system as well as in the metabolism could explain why some of the achievements gained in preclinical studies are lost in translation (Mestas and Hughes, 2004). Advances in the ability to evaluate human processes in preclinical murine models went hand in hand with a systematic progression of genetic modification to develop animal models transgenic for genes of interest in the pathogenesis of human diseases and immunodeficient host mice that could sustain growth of human cells and tissues and reproduce the histological features of related pathologies. Here, the evolution in preclinical murine model development will be described pointing out the main breakthroughs that led to the great progress of the last four decades.

MOUSE MODELS IN ONCOGENESIS AND CANCER THERAPY

Models to study human tumor physiology and pathobiology should reproduce the most clinically relevant features of human diseases. Their usefulness will depend on how close they replicate the initial phases of a human disease as well as histopathological features and then progress through the same

stages displayed by human pathologies showing similar systemic effects. From a molecular point of view, they should involve the same genes and signalling pathways detected in human pathology. Furthermore, in these models, drug metabolism, kinetics of action and potential side effects should be well simulated to assess the efficacy of new therapeutic agents. So far, mouse models used in preclinical research have not allowed to overcome the morphological and functional differences between mouse and human physiology and to accurately study the metabolic modifications of therapeutic agents in humans. All these limitations led to a growing need for animal models in which to carry out *in vivo* more accurate translational studies. Two main features to define a reliable model can be mentioned. The first is the generation of genetically modified murine models expressing oncogenes critical for human tumor development. These models have been instrumental in understanding the molecular mechanisms involved in tumor initiation but they have been less successful in replicating advanced cancers due to differences in transformation and progression between human and mouse (Cespedes et al., 2006). On the other hand, to overcome these constraints advances have been made in the development of immunodeficient murine models to investigate human tumor cell growth and metastasis. From the description of the *nude* phenotype in 1966 up to the generation of more stable and genetically defined immunodeficient phenotypes, huge advances in the study of antitumor approaches have been made. The growing efforts to develop reliable murine models led to the generation of preclinical models that could more closely sustain human cell proliferation, differentiation and physiological functions. These immunodeficient murine models lacking both adaptive and innate immunity could accept human tumor cell engraftment without the interference of the murine immune activity and are useful tools to investigate the function of antitumor non-immunological agents. In the following sections the main characteristics of the main murine models and research applications will be described.

Nude mice

The *nude* phenotype was firstly described by Flanagan in 1966. The *nu* locus was localized on chromosome 11 and the mutated gene named *Hfh11* (HNF-3/*fork head homologue 11*), is a member of the Fox gene family (Hirasawa et al., 1998). This mutation causes a pleiotropic phenotype: *nude* mice are characterized by loss of hair, thymic agenesis and deficiency of mature T lymphocytes. The related immune defects depend on the inability to produce cytotoxic helper or suppressor T lymphocytes. Because of the inefficient adaptive T compartment, B cell-mediated immune responses are also lowered and immunoglobulin (Ig) serum levels are altered (IgG and IgA serum levels are greatly reduced, while IgM levels are quite normal compared to immunocompetent mice). Since the definition of the immune alteration causing this phenotype, *nude* mice have been used as recipient of human tumor cell engraftment, to evaluate human tumor growth and spread and test *in vivo* the efficacy of new therapeutic approaches. However, *nude* mice show a partial immunodeficient phenotype, they still present a functional innate immune compartment, a complete natural killer (NK) activity and a partially impaired B cell functionality. Both NK and the other cells of the innate immunity (macrophages and granulocytes) could inhibit or at least control tumor growth and metastasization (Hanna and Burton, 1981; Lozupone et al., 2000). To overcome NK-dependent control of tumor cell behavior, *nude* mice can be treated with drugs or antibodies, as for example cyclophosphamide or anti-asialo GM1 antiserum to reduce NK cell content, prior to cell engraftment. However NK depletion is only temporary and not complete in all organs. Furthermore, evidences of T cell extrathymic maturation and B cell functionality in this mice reveal an unstable and not complete immunodepression that could interfere with human tumor growth and metastatic spread (Yui et al., 1988; Lake et al., 1991; Orengo et al., 2003).

Scid, NOD-scid and NOG mice

Even though *nude* mice could successfully sustain the engraftment of human tumor cells, normal cells are usually rejected. In 1983, Bosma and colleagues described a new mutant strain, with mutation in the *Prkdc*^{scid} gene (protein kinase, DNA activated, catalytic polypeptide; severe combined immunodeficiency, abbreviated *scid*). Due to the mutation in a gene responsible for DNA repair, recombination of variable, diversity and joining (VDJ) segments necessary for the generation of T- and B-cell receptors is severely impaired. The resulting phenotype includes a combined immunodeficiency due to the absence of both mature B and T cells besides a strong radiation sensitivity due to the genetic defect in genes involved in double-strand break repair. However these mice (like *nude* mice) still present a functional innate immune system (NK cells, macrophages and neutrophils). The DNA repair defect is not only affecting TCR and immunoglobulin rearrangement but is a general defect of all cell types resulting in a relatively high sensitivity to DNA damaging agents (i.e., x-irradiation, bleomycin). Furthermore, in addition to the gene responsible for *scid* phenotype, other genes (among which the recombinant activating genes, RAG1 and RAG2) are involved in VDJ recombination. A consequence of the incomplete block of VDJ recombination is that a number of mice show a "leaky" phenotype (i.e., residual T and B activity with measurable levels of serum Ig). The leakiness increases with age and antigen exposure and virtually all *scid* mice have detectable T and B cells at 1 year of age (Hendrickson, 1993). The availability of *scid* mice provided an alternative model for studying human tumors *in vivo*. When direct comparisons between *nude* and *scid* mice have been made, diverse propensities of human tumor cell growth and metastasization have been reported (Williams et al., 1993; Garofalo et al., 1993). Unlike *nude* mice, *scid* mice could accept the engraftment of hematopoietic cells and still now represent the main murine model to study human leukemia cells behavior and treatment strategies.

However, levels of engraftment were still low and, due to their high radiation susceptibility, *scid* mice showed a high propensity to develop spontaneous tumors and could not be used to test *in vivo* antitumor agents that act on DNA metabolism or have a DNA-damaging activity causing double-strand breaks. To overcome the above reported limitations, and abrogate, at least partially, the functionality of the innate immune system, different murine strains displaying other immunodeficiencies were obtained, e.g. through crossing *scid* mice with non-obese diabetic (NOD) mice.

NOD mice display several abnormalities in the immune system, which cooperate to determine the diabetes susceptibility phenotype (Makino et al., 1980). The immunodepressed phenotype of NOD mice is caused by at least three different mutated genes (located on the so called Idd loci on Chromosome 3, 9 and 17, but more than 20 loci have been identified even not well characterized) important for antigen presentation and T-cell function. The resulting immune phenotype is characterized by reduced NK activity and T-cell count. Furthermore the innate immunity is impaired due to the absence of macrophages and circulating complement. Other immune defects affected the differentiation and function of antigen presenting cells (Shultz et al., 1995; Anderson and Bluestone, 2005).

Crossing *scid* mice with non-obese diabetic (NOD) mice led to the generation of NOD-*scid* mice. NOD-*scid* mice displayed all immune defects illustrated for *scid* and NOD mice, but unlike NOD mice they did not develop diabetes. Unfortunately NOD-*scid* mice still show a certain degree of radiation sensitivity and have a decreased life span (no more than eight months) due to the development of thymic lymphomas. Moreover, they still present a residual innate immunity activity, and a low but still present NK cell activity.

Several murine models with defined genotype defects have been established acting on genes with a key role in the functionality of the innate immune compartment that impedes the successful engraftment of human normal and tumor cell lines or tissues (i.e., genes involved in the lysosomal

activity, perforin release or β2-microglobulin expression) (Thomsen et al., 2005; Shultz et al., 2007).

In 2002 NOD/SCID/γc^{null} (NOG) murine model was first described (Ito et al., 2002). The Interleukin-2 receptor (IL-2R) γ common chain is a crucial subunit of hetero di- or trimeric high-affinity receptors for different cytokines (IL-2, IL-4, IL-7. IL-9, IL-15 and IL-21), and it is required for signalling through these receptors. All these cytokines are responsible for the development and homeostasis of B, T and NK lymphocytes. IL-2R γ knockout mice are characterized by the absence of functional NK cells, the reduction in B and T cell number and show other abnormalities such as very small thymus, mesenteric lymph node and spleen due to severe hypoplasia (Cao et al., 1995; Di Santo et al., 1995). NOG mice obtained by crossing NOD-*scid* with γ common knockout mice, show no mature B and T lymphocytes and are radiation sensitive like NOD-*scid* mice, but also completely lack NK cells and the functionality of the innate immunity is further reduced. Moreover, NOG mice do not show the development of spontaneous lymphomas, likely due to the lack of cytokine signalling through the IL-2R γ chain and show a normal life span (Shultz et al., 2005; Ito et al., 2008).

Rag2$^{-/-}$;γc$^{-/-}$ mice

As previously described, RAG genes encode two recombination activating proteins, RAG-1 and RAG-2, whose cellular expression is restricted to lymphocytes during their developmental stages and is fundamental for the development of mature B and T lymphocytes. In 1992, Shinkai and colleagues developed a RAG-2-deficient murine model characterized by the absence of functional B and T cells, similarly to *scid* mice (Shinkai et al., 1992). Unlike *scid* mutation, due to the specificity of RAG function and its involvement in the initial phases of VDJ rearrangement, the resulting phenotype is completely stable and affects only lymphocyte maturation. The deletion of RAG-2 activity

caused several alterations in the physiology of the immune system: mice showed decreased or occasionally absent thymus, and a reduction of the cellularity of other lymphoid organs as spleens and lymph nodes. Crossing of RAG-2- and γ common chain-deleted mice led to the generation of a completely genotipically defined immunodepressed murine model named $Rag2^{-/-};\gamma c^{-/-}$ mice. As previously reported, the common cytokine receptor γ chain is a high and median affinity component of the receptors for several cytokines, such as IL-2, involved in the activation of T and NK cells and in control of the peripheral tolerance, IL-7 responsible for the development and functionality of lymphocytes, IL-9 that acts as a growth factor for mast cells, IL-15 involved in the regulation of the development and maturation of NK cells and the homeostasis of memory lymphocytes, IL-4 and IL-21 that act on immunoglobulin production and regulate the isotypic switch. $Rag2^{-/-};\gamma c^{-/-}$ mice show a combined immunodeficiency characterized by the complete absence of B, T and NK cells and share all the properties described for NOG mice, with the exception that they are completely resistant to radiations (Shultz et al., 2007).

$Rag2^{-/-};\gamma c^{-/-}$ mice and NOG mice represent the most innovative animal model for *in vivo* studies on the development of human immune system. So far the best humanization results after engraftment with human hematopoietic cells were obtained in these two murine models and more possibilities to study human immune responses to immunological approaches are under investigation (Manz, 2007; Shultz et al., 2007). A few recent data also support that these mice could be more permissive models for *in vivo* studies on tumor biology and therapy (Suemizu et al., 2007; Le Devedec et al., 2009; Bertilaccio et al., 2009).

SECTION 1

GROWTH AND METASTATIC SPREAD OF HUMAN TUMOR CELL LINES IN IMMUNODEFICIENT MURINE MODELS

INTRODUCTION

Transplantable models

One of the main advances in cancer research was the study of human tumor cell lines and tissues that could be grown in preclinical models. Metastases still remain the most relevant problem in cancer therapy. A preclinical murine model should be metastatic, allowing tumor growth and spread with a pattern of organ colonization similar to that of the original human tumor (Cespedes et al., 2006).

Transplantable models to study the growth and metastatic behavior of tumor cells can be divided into two main groups, syngeneic models or xenograft models (Khanna and Hunter, 2005). The former model is referred to murine (mouse or rat origin) cancer cell lines that can give rise to tumors once implanted in inbred animals with the same genetic background. This type of experimental model allows the evaluation of tumor-host interactions that may contribute to the metastatic propensity. However, this model system is strictly related to murine metabolism and lack many features of human tumors due to species-specific differences in oncogenesis. Human-mouse xenograft models consist of human cancer cell lines grown in immunocompromised murine hosts. In this case, use of immunodeficient mice to prevent immune rejection rules out the possibility to examine the role of the immune system in control of tumor growth and metastasis. However, human tumor cells can interact with murine stromal cells. The residual immune activity of murine models could prevent or

reduce human tumor growth and, as mentioned, this constraint led to the development of more severely immunocompromised murine models.

Growth and metastasis of transplantable tumor models, can be studied through injection in different anatomical sites. Subcutaneous injection was the most used way, but tumors grow in a heterotopic site. The administration in the same anatomical location from which the injected tumor was derived is referred to as orthotopic delivery. Metastatic ability of transplantable tumors can also be tested through direct injection in the systemic circulation; metastases so obtained are frequently referred to as experimental metastases. Lateral vein injection results primarily in pulmonary metastases, intrasplenic or portal vein injection are reliable approaches to induce liver metastases, intracardiac injection of cells may result in metastases in a broad panel of sites including bone. Other metastatic nodules may be detected resulting from the specific interaction between tumor cells and host determinants (i.e., stromal cell peculiarity, organ tropism, and specific sensibility to growth or chemotactic factors). Experimental metastasis model provide several advantages because of generally rapid time of metastasis induction, reproducibility and control of the number of injected tumor cells. However, i.v. injected tumor cells skip the early steps in the metastatic cascade (i.e., acquisition of a transformed phenotype, invasion of the surrounding stroma and extravasation into the hematopoietic system). After subcutaneous injection of human tumor cells in xenogeneic models, metastases were rarely detected and the heterotopic setting was often referred as “non-metastatic” setting. The use of orthotopic injection more closely reproduces human cancer behavior including tumor vascularity, metastatic organ propensity and responsiveness to chemotherapy (Bibby, 2004; Khanna and Hunter, 2005). Interestingly, in some cases changing the injection site (orthotopic vs heterotopic site) resulted not only in different growth rates and metastasization patterns but also in a differential sensitivity to therapeutic agents, revealing the importance of tumor cell-stroma cell interaction in the behavior of tumor cells (Wilmanns et al., 1992; Cespedes et al., 2006).

Comparative studies of human tumor behavior

Nude and *scid* mice have been so far the most used murine models to study human tumor behavior. Several studies reported growth of human tumor cell lines in these immunodepressed murine models and the detection of metastatic dissemination of tumor cells. Comparative studies with different immunodepressed mice have been performed. Garofalo and colleagues compared the metastatic ability of different tumor histotypes (melanoma, colon, ovarian and renal cancer) in *nude*, *scid* and *beige/nude/xid* mice (Garofalo et al., 1993). Tumor growth and metastatic ability seemed to correlate not only with the severity of the immunodepression of investigated murine models but several data revealed that different tumor types showed different propensities also linked to intrinsic characteristics of tumor cell lines (Garofalo et al., 1993; Welch et al., 1997). The orthotopic implantation of a broad panel of human tumor cells of different histotypes in *nude* mice showed metastatic patterns similar to those observed in humans (Cespedes et al., 2006). Nevertheless, the innate immune components still present in *nude* and *scid* murine models (i.e., granulocytes and NK cells) could control human tumor growth and metastasization propensity. *Scid* or NOD-*scid* mice treated with anti-NK antibodies (i.e., GM1 antiserum or TMβ-1) or antibodies to deplete granulocytes (i.e., RB6-8C5) showed an increased level in tumor metastasis (Lozupone et al., 2000), but higher levels were obtained in more profoundly immunodepressed NOG mice (Dewan et al., 2005). Other recent studies in NOG mice showed the efficiency of severely immunodepressed murine models in allowing human tumor growth leading to a broader and more efficient metastasization. Suemizu and colleagues demonstrated the generation of a reliable model of liver metastasization of human pancreatic carcinoma in NOG mice, while other murine models (namely, *nude* and *scid* mice) showed low metastatic rates despite the large number of intrasplenic injected cells (Suemizu et al., 2007).

AIM

This part of the work aimed at studying the metastatic phenotype and mechanism of human sarcoma and other types of human tumor cell lines in the severely immunodepressed $Rag2^{-/-};\gamma c^{-/-}$ mice lacking T, B and NK immunity. The possible use of $Rag2^{-/-};\gamma c^{-/-}$ murine model to evaluate *in vivo* new antitumor and antimetastatic non immunological strategies was also addressed.

RESULTS

Metastatic spread of human sarcoma cell lines in Rag2$^{-/-}$;γc$^{-/-}$ mice

Previous data showed that human rhabdomyosarcomas, osteosarcomas and Ewing's sarcomas cell lines had a poor metastatic ability in *nude* mice, even after the intravenous injection in NK-depleted hosts.

The metastatic ability of a panel of human musculoskeletal sarcomas was tested through intravenous (i.v.) injection in Rag2$^{-/-}$;γc$^{-/-}$ mice (Table 1). *Nude* mice with NK depletion obtained through anti-asialo GM1 antiserum were also used as control model. Human sarcoma cell lines comprised osteosarcoma (Saos-2 and U2OS), Ewing's sarcoma (TC-71 and 6647), and rhabdomyosarcoma (the alveolar rhabdomyosarcoma cell line SJ-Rh4 and two clones derived from the RD embryonal rhabdomyosarcoma cell line, RD/12 and RD/18).

In a first set of experiments mice received the intravenous injection of 2 x 10^6 human tumor cells. Several sarcoma cell lines showed a 100% incidence of lung metastases (Saos-2, U2-OS, TC-71, 6647 and RD/18) in Rag2$^{-/-}$;γc$^{-/-}$ mice with a significant increase in the median number of metastases compared to *nude* mice (Table 1). Furthermore, the metastatic growth was faster in Rag2$^{-/-}$;γc$^{-/-}$ than in *nude* mice as evidenced by the earlier median time to sacrifice in the double knockout host compared to *nude* mice (Table 1). Interestingly, most sarcoma cell lines (Saos-2, TC-71, 6647, SJ-RH4, RD/12 and RD/18) showed a very high metastatic colonization of the liver in Rag2$^{-/-}$;γc$^{-/-}$ mice, while completely failed to colonize the liver of *nude* mice (Table 1).

Table 1. Metastatic capacity of human sarcoma cells in immunodepressed mice

Cell line	i.v. cell dose	Mice	Median time to sacrifice (days)	Lung metastases				Liver metastases				Other metastatic sites			
				Incidence	%	Median	Range	Incidence	%	Median	Range	Incidence	%	Median	Range
Saos-2	$2x10^6$	Rag2/γc-KO	68	4/4	100	**>200***	>200->200	4/4*	100	**>200***	>200->200	4/4*	100	**3***	1-3[2]
	$2x10^6$	Rag2/γc-KO	48	5/5[#]	100	**>200***	>200->200	5/5*	100	**7***	2-17	0/5	0	**0**	0-0
	$2x10^6$	nude[1]	89	8/19	42	**0**	0-161	0/19	0	**0**	0-0	0/19	0	**0**	0-0
U2-OS	$2x10^6$	Rag2/γc-KO	36	9/9	100	**>200***	>200->200	0/9	0	**0**	0-0	0/9	0	**0**	0-0
	$2x10^6$	nude[1]	63	17/20	85	**41**	0->200	0/20	0	**0**	0-0	0/20	0	**0**	0-0
TC-71	$2x10^6$	Rag2/γc-KO	30	12/12*	100	**37***	1-105	12/12*	100	**>200***	>200->200	8/12	67	**1**	0-6[2]
	$2x10^6$	nude[1]	78	8/24	33	**0**	0-10	0/24	0	**0**	0-0	16/24	67	**1**	0-3[2]
6647	$2x10^6$	Rag2/γc-KO	21	5/5	100	**114***	18-157	5/5*	100	**>200***	>200->200	5/5	100	**21***	13-29[2]
	$2x10^6$	nude[1]	54	13/20	65	**5**	0-67	0/20	0	**0**	0-0	18/20	90	**3**	0-16[2]
SJ-RH4	$2x10^6$	Rag2/γc-KO	59	1/5	20	**0**	0-4	5/5	100	**>200**	>200->200	2/5	40	**0**	0-2[2]
	$2x10^6$	Rag2/γc-KO	35	0/11	0	**0**	0-0	11/11	100	**40**	14-113	4/11	36	**0**	0-2[2]
	$2x10^6$	nude[1]		*n.d.*				*n.d.*				*n.d.*			
RD/12	$2x10^6$	Rag2/gc-KO	79	11/14*	79	**3***	0-17	14/14*	100	**75***	25-218	10/14*	71	**2***	0-3[2]
	$2x10^6$	nude[1]	97	6/19	32	**0**	0-8	0/19	0	**0**	0-0	0/19	0	**0**	0-0
RD/18	$2x10^6$	Rag2/γc-KO	36	15/15	100	**>200***	112->200	15/15*	100	**>200***	>200->200	15/15	100	**6***	4-57[2]
	$2x10^6$	nude[1]	58	14/15	93	**26**	0-327	0/15	0	**0**	0-0	15/15	100	**2**	1-8[2]

$Rag2^{-/-};\gamma c^{-/-}$ and *nude* mice received the intravenous injection of $2x10^6$ sarcoma tumor cells. [1]Pretreated with anti-NK antibodies (*see* Materials and methods).

[2]Metastatic sites: Saos-2: kidneys, adrenals, lymphoid organs; TC-71: kidneys, adrenals, bone, ovary; 6647: kidneys, adrenals, ovary, brown fat, lymphoid organs; SJ-Rh4: kidneys; RD/12: kidneys, adrenals, ovary; RD/18: kidneys, adrenals, ovary, lymphoid organs. $Rag2^{-/-};\gamma c^{-/-}$ indicated as Rag2/γc-KO. *n.d.*=not done.

Significance of difference versus *nude* mice: # $p<0.05$, * $p<0.01$ (χ_2 test for frequency, nonparametric Mann-Whitney rank sum test for metastasis number).

Liver metastases were the result of organ-selective homing of individual tumor cell lines, independent of the ability to colonize the lungs or other organs (Table 1 and Figure 1). As graphically reported in Figure 1, different sarcoma cell lines showed diverse organ-specific metastatic propensities for liver and lung colonization. Furthermore, several cell lines showed a metastatic propensity to colonize organs other than lungs and liver significantly enhanced in $Rag2^{-/-};\gamma c^{-/-}$ mice (see data for two rhabdomyosarcoma, RD/12 and RD/18 and Ewing's sarcoma, 6647 or osteosarcoma cell lines Saos-2 in Table 1). Metastatic sites were mainly kidneys, adrenal glands and lymphoid organs (see Table 1 for details). Multi-organ metastatic pattern was also observed after injection of decreasing cell numbers (data not shown).

These results indicated that $Rag2^{-/-};\gamma c^{-/-}$ mice were a more permissive host to study the metastatic behavior of human cell lines.

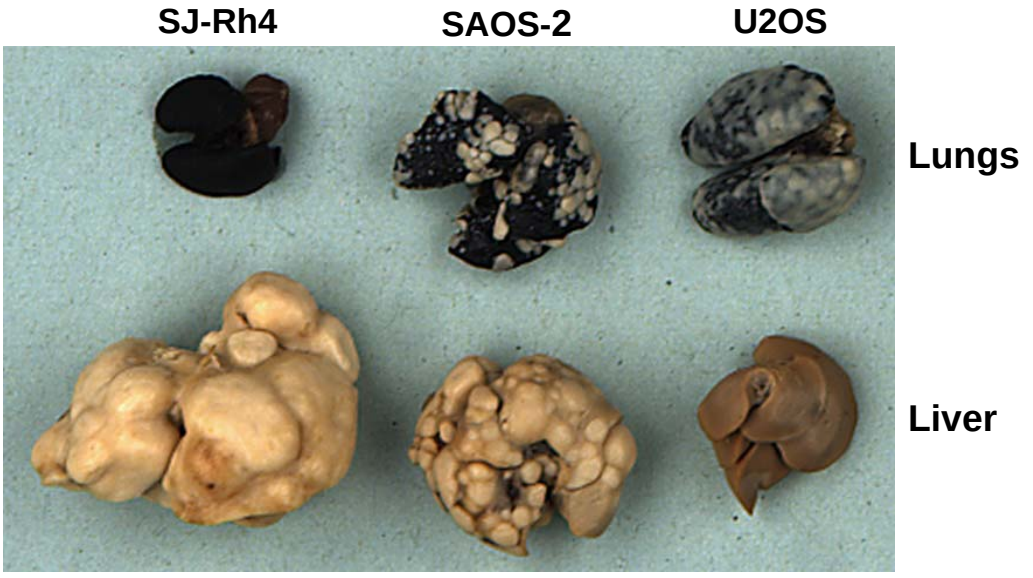

Figure 1. Organ-specific metastatic capacity of human sarcoma cells injected i.v. in $Rag2^{-/-};\gamma_c^{-/-}$ mice. Lungs (above, filled with black India ink to better identify and quantify metastases) and liver (below) of mice which had received the i.v. injection of indicated human sarcoma cells.

Imaging of Ewing's sarcoma metastasis

Ewing's sarcoma in humans usually show a strong tendency to metastasize to lungs and bones. TC-71 Ewing's sarcoma cell line is able to give rise to bone metastases in *nude* mice (Scotlandi et al., 2000). Because of the early sacrifice time due to liver colonization, in $Rag2^{-/-};\gamma c^{-/-}$ mice, bone metastases were hardly detectable. To obtain a more sensitive detection of metastases, TC-71 cells were transfected with pEGFPN1 plasmid to stably express Enhanced Green Fluorescent protein (EGFP). As illustrated in Figure 2, through fluorescent imaging, metastases to ovary and bones were observed, thus confirming that Ewing's sarcoma cells maintain such tropism in $Rag2^{-/-};\gamma c^{-/-}$ mice.

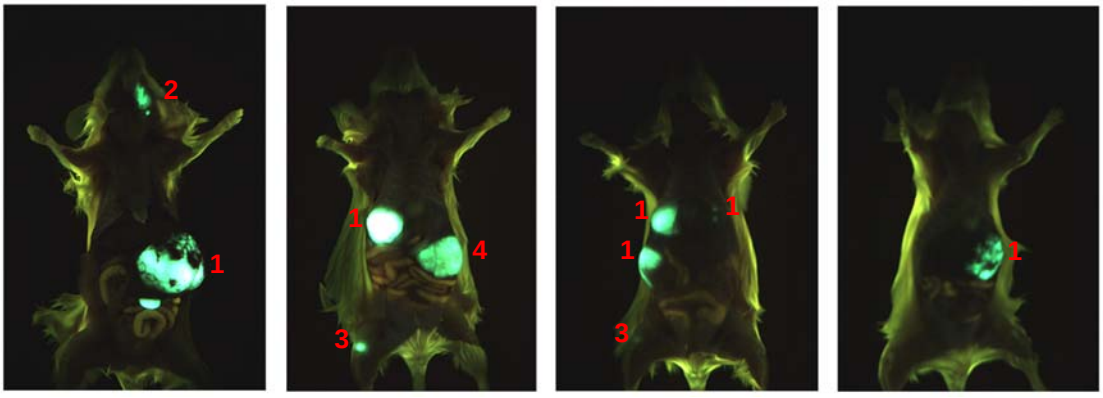

Figure 2. Organ-specific metastatic ability of human Ewing's sarcoma cells injected i.v. in $Rag2^{-/-};\gamma c^{-/-}$ mice. TC-71 EGFP cells were i.v. injected in $Rag2^{-/-};\gamma c^{-/-}$ mice and metastases were localized with a fluorescent imaging device (see Materials and Methods for details). 1: liver metastases, 2: mandible metastases, 3: femur metastases; 4: ovary metastases.

Tumorigenicity and spontaneous metastases in Rag2$^{-/-}$;γc$^{-/-}$

Rag2$^{-/-}$;γc$^{-/-}$ mice showed the appearance of spontaneous metastases after the subcutaneous (s.c.) injection of human osteosarcoma (U2-OS) and Ewing's sarcoma (TC-71) cell lines (Table 2). The s.c injection of a lower amount of tumor cells in Rag2$^{-/-}$;γc$^{-/-}$ mice than in *nude* mice is sufficient to allow the local growth of tumor and the induction of spontaneous metastases to the lungs. Rag2$^{-/-}$;γc$^{-/-}$ mice showed a faster tumor growth than *nude* mice, and interestingly, at the time of sacrifice, metastases in organs other than lungs were detectable in both investigated human sarcoma cell lines (Table 2).

Table 2. Tumorigenicity and induction of spontaneous metastasis of human sarcoma cells

Cell line	s.c. cell dose	Mice	Median time to sacrifice (days)	Tumorigenicity			Lung metastases				Liver metastases				Other metastatic sites			
				Incidence	%	Median latency (days)	Incidence	%	Median	Range	Incidence	%	Median	Range	Incidence	%	Median	Range
U2-OS	$10x10^6$	Rag2/γc-KO	78	5/5	100	31	5/5	100	**>200***	>200->200	2/5	40	**0**	0-1	$4/5^{\#}$	80	**3**$^{\#}$	0-4[1]
	$30x10^6$	Nude	118	4/5	80	33	2/5	40	**0**	0-45	0/5	0	**0**	0-0	0/5	0	**0**	0-0
TC-71	$2x10^6$	Rag2/γc-KO	27	5/5	100	10	3/5	60	**3**	0-14	0/5	0	**0**	0-0	3/5	60	**1**	0-1[1]
	$3x10^6$	Nude	42	5/5	100	8	0/5	0	**0**	0-0	0/5	0	**0**	0-0	0/5	0	**0**	0-0

[1]Metastatic sites: U2-OS: kidneys, adrenals, peritoneum; TC-71: lymph nodes.

$Rag2^{-/-};\gamma c^{-/-}$ mice are indicated as Rag2/γc-KO.

Significance of difference versus *nude* mice: # $p<0.05$, * $p<0.01$ (χ_2 test for frequency, nonparametric Mann-Whitney rank sum test for metastasis number)

Metastatic ability of human carcinoma cell lines in Rag2$^{-/-}$;γc$^{-/-}$ mice

To investigate whether the high metastatic ability and mostly the liver metastatic colonization observed in Rag2$^{-/-}$;γc$^{-/-}$ mice were a peculiar characteristic of the investigated sarcoma cell lines or a general feature of all types of human tumors, a panel of carcinoma cell lines of diverse histologic origin (liver, colorectal, breast, or ovary) was studied. Data obtained after the i.v. injection of 2 x 10^6 cells, in analogy with the experiments of sarcoma metastasization, are reported in Table 3. All investigated human carcinomas were completely unable to metastasize to the liver of Rag2$^{-/-}$;γc$^{-/-}$ mice (Table 3), irrespective of their ability to colonize the lungs or other mouse organs. Liver carcinoma cells (Hep-G2) were able to metastasize to different lymphoid organs, adrenal glands, kidney and urogenital system. Ovary carcinoma cells (SK-OV-3) and the colon adenocarcinoma cell line HT-29 showed essentially only lung metastases (Table 3). A comparison of metastatic spread obtained in Rag2$^{-/-}$;γc$^{-/-}$ and *nude* mice was made with SK-OV-3 cells, confirming that Rag2$^{-/-}$;γc$^{-/-}$ mice allow a better detection of metastatic ability (Table 3).

Table 3. Metastatic capacity of human carcinoma cells in Rag2$^{-/-}$;γc$^{-/-}$ mice

Cell line	i.v. cell dose	Mice	Median time to sacrifice (days)	Lung metastases				Liver metastases				Other metastatic sites			
				Incidence	%	Median	Range	Incidence	%	Median	Range	Incidence	%	Median	Range
HepG2	2x10^6	Rag2/γc-KO	43	0/5	0	**0**	0-0	0/5	0	**0**	0-0	5/5	100	**6**	4-11[2]
Caco-2	2x10^6	Rag2/γc-KO	97	0/4	0	**0**	0-0	0/4	0	**0**	0-0	0/4	0	**0**	0-0
HT-29	2x10^6	Rag2/γc-KO	19	5/5	100	**>200**	>200->200	0/5	0	**0**	0-0	0/5	0	**0**	0-0
MCF7	2x10^6	Rag2/γc-KO	97	1/4	25	**0**	0-1	0/4	0	**0**	0-0	0/4	0	**0**	0-0
SK-OV-3	2x10^6	Rag2/γc-KO	37	3/3	100	**>200**	>200->200	0/3	0	**0**	0-0	0/3	0	**0**	0-0
	2x10^6	nude[1]	61	4/4	100	**32**	5->200	0/4	0	**0**	0-0	0/4	0	**0**	0-0

[1]Pretreated with anti-NK antibodies (*see* Materials and methods).

[2]Metastatic sites: kidneys, adrenals, urogenital system, lymphoid organs.

Rag2$^{-/-}$;γc$^{-/-}$ mice are indicated as Rag2/γc-KO..

In vitro analysis of molecular mechanisms involved in metastatic growth

Liver metastatic localizations observed in $Rag2^{-/-};\gamma c^{-/-}$ mice, specific of most sarcoma cell lines, and the high rate of metastatic growth in this organ, could be due to the presence in liver of factors able to induce proliferation or chemotaxis of sarcoma cells. As a model of interaction with liver microenvironment, tumor cells were exposed to the culture supernatant of a liver-derived human hepatoma cell line (HepG2 cells). Figure 3 shows that the growth of sarcoma cell lines (with the exception of U2-OS cells which were not able to colonize the liver) was stimulated by HepG2 conditioned medium. For these sarcoma cell lines, the growth ability in conditioned medium was significantly increased compared with the growth observed in normal medium. Growth of carcinoma cells, which did not show any hepatic metastasization propensity, was not modified by conditioned medium (Figure 3).

A key mechanism relevant for metastatic spread and organ-selective colonization is the ability of tumor cells to respond to chemotactic stimuli. Experiments to evaluate the migratory propensity of sarcoma cells in medium conditioned by hepatic cells compared to normal medium were performed. Each sarcoma cell line showed a different intrinsic basal migratory capacity (Figure 4A). After exposure to HepG2 conditioned medium, the migratory ability was stimulated in same sarcoma cell lines (Saos-2 and RD/12 showed a significant increase in the number of migrated cells) (Figure 4A). SJ-Rh4 and RD/18 cell lines (whose growth was stimulated by HepG2 supernatants) showed a very low basal migratory propensity that was not stimulated by conditioned medium. Similar migratory patterns were obtained after exposure of sarcoma cells to medium conditioned by mouse liver progenitor cells (MLP29.1C cell line) (data not shown). Carcinoma cells did not show any migratory ability both in normal and conditioned medium (Figure 4A).

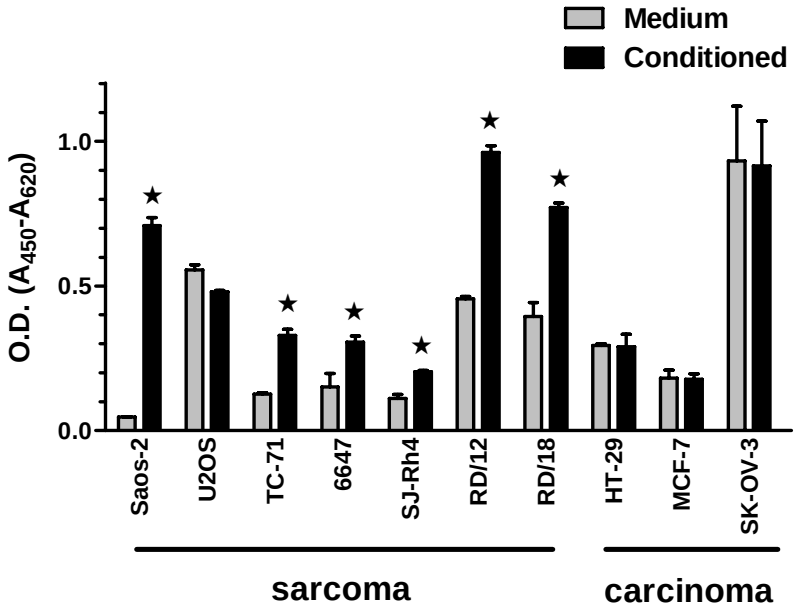

Figure 3. In vitro growth of human sarcoma and carcinoma cells exposed to medium conditioned by human liver-derived cells (HepG2). 10^4 cells were seeded in 96 well plates in normal or conditioned medium. Cell growth was evaluated after 48 hours of culture through the WST-1 test (*see* Materials and Methods for details). Data are means of at least two independent experiments. Stars indicate a statistically significant difference between normal and conditioned medium ($p<0.05$ at least, Student's *t* test).

The liver produces a wealth of growth factors that could attract tumor cells and make them proliferate, which could be one of the reasons why it is a frequent site of metastatic spread in $Rag2^{-/-};\gamma c^{-/-}$ mice. One logical link between the liver and sarcomas is the insulin-like growth factor (IGF) axis. The liver is the major producer of IGFs in the body (Miyamoto et al., 2005; Rikhof et al., 2009). Several studies demonstrated that human sarcoma tissues and cell lines express type 1 IGF receptor (IGF1R) which mediates responses to both IGF1 and IGF2 and underline the involvement of related signalling circuits in human

sarcoma cell growth, in particular rhabdomyosarcomas and Ewing's sarcomas, in a paracrine and/or autocrine manner (Scotlandi and Picci 2008; Rikhof et al., 2009).

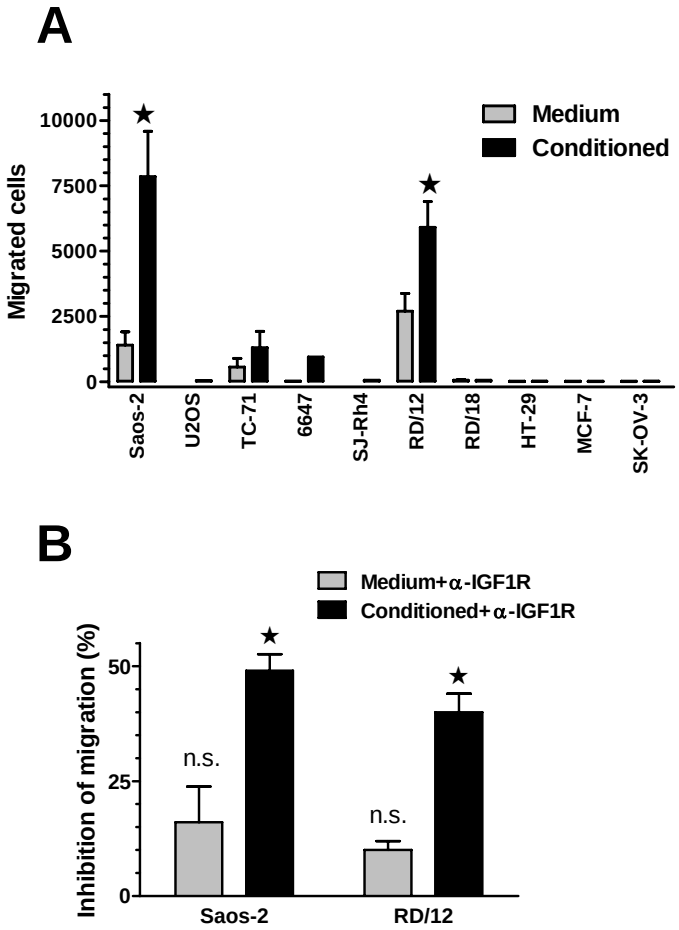

Figure 4. Migration of human sarcoma cells in conditioned medium. A: Migration assay. Star indicates a significant difference between normal and conditioned medium ($p<0.05$ at least, Student's *t* test). **B**: Percentage of reduction of liver-stimulated or basal migration caused by IGF1R neutralization. Star or n.s. indicate a significant or not significant difference of cell migration versus cell migration in the corresponding medium in the absence of anti-IGF1R, respectively (star = $p<0.05$ at least, Student's *t* test; n.s. = not significant).

To evaluate the involvement of IGFs in the chemotactic behavior induced by HepG2 conditioned medium in sarcoma cells, migration experiments were performed in which a blocking monoclonal antibody against IGF1R was added to the conditioned or not conditioned medium. Figure 4B shows that the blocking of IGF1R reduced by half the chemotactic activity of HepG2 conditioned medium, thus demonstrating a significant contribution of hepatocyte-derived IGFs in sarcoma migration ability.

Therapy of liver metastasis by dual PI3K-mTOR inhibitor

The broad liver metastasization of human sarcoma cell lines in $Rag2^{-/-};\gamma c^{-/-}$ mice will allow the employment of this murine model to study new non-immunological antimetastatic approaches. In particular, the involvement of the IGF axis in the liver tropism of human sarcoma cells suggests that therapeutic agents targeting IGF1R or downstream signal transducers could have a specific antimetastatic effect in this system. To test this hypothesis, $Rag2^{-/-};\gamma c^{-/-}$ mice received the i.v. injection of the rhabdomyosarcoma cell line RD/18 to induce liver micrometastases. The day after and for a total of 18 administrations, tumor cell injected mice received the treatment with the dual PI3K/mTOR kinase inhibitor NVP-BEZ235 (Serra et al., 2008). As clearly illustrated in Figure 5A, mice treated with NVP-BEZ 235 showed a striking reduction in the liver metastatic burden. The liver weight of treated mice reached values similar to those of naïve $Rag2^{-/-};\gamma c^{-/-}$ mice, due to a statistically significant reduction of the metastatic load (Figure 5B). The numbers of metastases in lungs and other sites were also significantly decreased (Figure 5B). This result illustrated the experimental reliability of $Rag2^{-/-};\gamma c^{-/-}$ mice model to test the metastatic ability of human tumor cells.

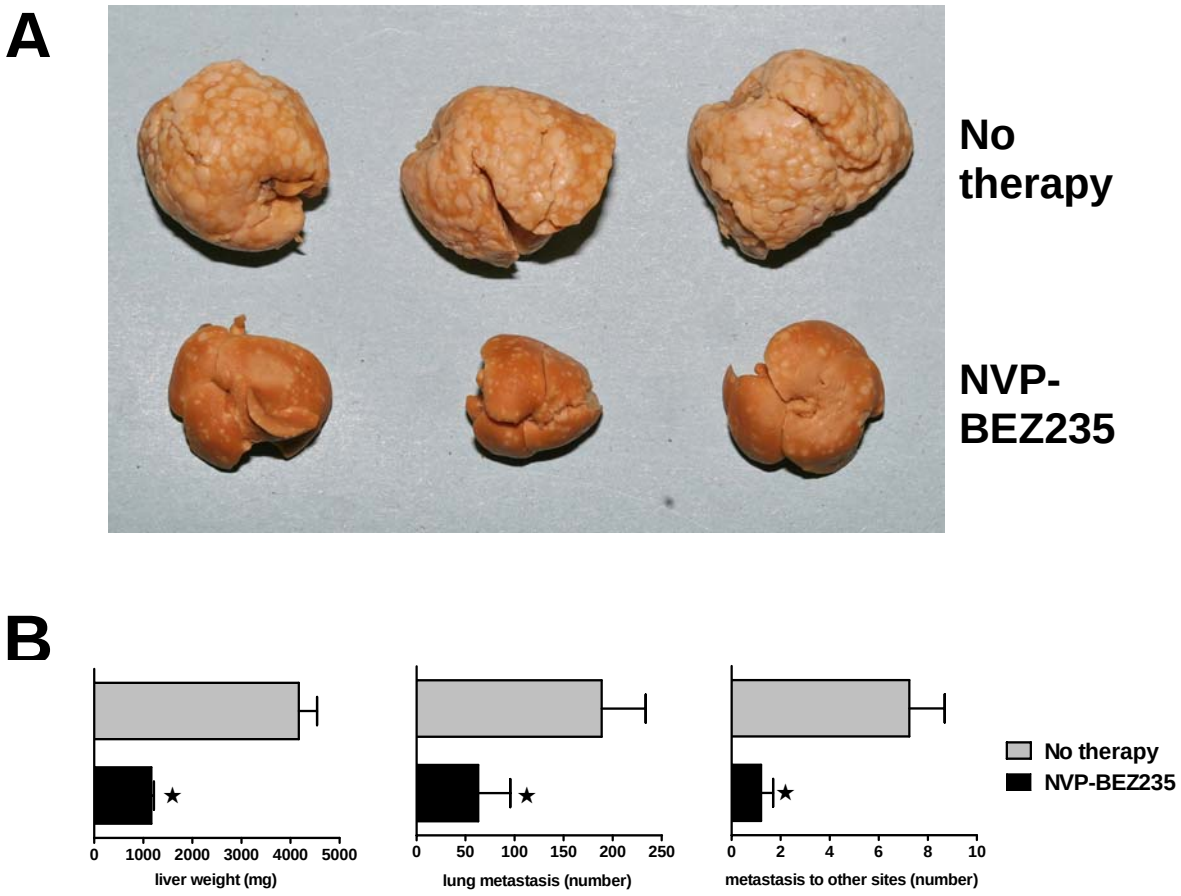

Figure 5. Therapy by NVP-BEZ235 of liver metastases induced by the i.v. injection of human RD/18 rhabdomyosarcoma cells in Rag2$^{-/-}$;γc$^{-/-}$ mice. A: liver metastatic burden in untreated mice (above) or in mice treated with NVP-BEZ235 (below). **B**: Quantitative evaluation of metastatic load to liver (left), lungs (middle), and other sites (right). Mean and standard error of the mean (SEM) from 5 mice is shown for each group. Star indicates a statistically significant difference ($p<0.05$, Student's *t* test). Liver weight in normal Rag2$^{-/-}$;γc$^{-/-}$ mice was 1133 ± 30 mg.

The results reported above have been included in a paper accepted for publication (*Nanni et al., Eur J Cancer 2009 Dec 21 E-pub*).

Metastatic behavior of rhabdomyosarcoma cells engineered to improve myogenic differentiation

Due to high tumor growth and metastatic spread of sarcoma cells in this model, $Rag2^{-/-};\gamma c^{-/-}$ mice were used as murine model to test *in vivo* the therapeutic efficacy of a gene therapy approach. Rhabdomyosarcoma cell populations revealed a considerable differentiative heterogeneity, containing a variable proportion of cells at different stages of differenziation. The overall proliferative and tumorigenic capacity of rhabdomyosarcomas is inversely related to the degree of myogenic differentiation (Lollini et al., 1991; Merlino and Helman, 1999). Myogenin (Myog) is one of the member of the myogenic regulatory factors that coordinate gene expression and drive terminal muscle differentiation (De Giovanni et al., 2009) characterized by the expression of myosin and other contractile proteins. RD/12 cells show a very low expression of myogenin and a low differentiation capability. Preliminary *in vitro* experiments, showed that the forced expression of Myog gene in RD/12 cells (RD/12-Myog cells) led to an overall increased myosin production, to the appearance of a fraction of multinuclear myotube-like elements and decreased the migratory capacity (Astolfi et al., 2001 and data not shown).

The therapeutic implication of the forced Myog expression was analyzed both in experiments of tumor growth and in experiments of metastatic ability after i.v. injection in *nude* and $Rag2^{-/-};\gamma c^{-/-}$ mice. The tumorigenicity of RD/12-Myog cells in *nude* mice was strongly impaired with respect to RD/12 or RD/12 cells transduced with an empty plasmid (RD/12-Neo cells) (data not shown). Studies of induction of experimental metastasization in *nude* mice revealed that only 35% of mice were affected by lung nodules after the injection of both RD/12 or RD/12-Myog cells. As reported above, $Rag2^{-/-};\gamma c^{-/-}$ mice injected with RD/12 cells showed both lung and liver colonization (Table 1 and Table 4). When i.v. injected in $Rag2^{-/-};\gamma c^{-/-}$ mice, RD/12-Myog cells gave a reduced liver metastatic load compared to RD/12 or RD/12-Neo cells (Table 4

and Figure 6). This experiment confirms the translational potential of $Rag2^{-/-};\gamma c^{-/-}$ mice as an efficient murine model to investigate the behavior of human tumor cell lines and to evaluate antitumor approaches.

Table 4. Metastatic spread of myogenin-transduced human rhabdomyosarcoma cells in $Rag2^{-/-};\gamma c^{-/-}$ mice.

Cells	Lung metastasis			Liver metastasis		
	Incidence	Median	Range	Incidence	Median	Range
RD/12	3/5 (60%)	2	0-17	5/5 (100%)	125	74-218
RD/12-Neo2	3/4 (75%)	2	0-2	4/4 (100%)	134	109-29
RD/12-Myog1	4/5 (80%)	2	0-31	5/5 (100%)	30*	23-36
RD/12-Myog2	2/4 (50%)	1	0-1	4/4 (100%)	60*	32-89

$Rag2^{-/-};\gamma c^{-/-}$ mice received the i.v. injection of 2 x 10^6 cells and were sacrificed 10 weeks later.
* indicates a significant difference versus RD/12 and RD/12-Neo2 ($p<0.05$, Wilcoxon rank sum test).

Figure 6. Metastatic spread of RD/12 and RD/12-Myog cells to the liver of Rag2$^{-/-}$;γc$^{-/-}$ mice. Three representative fixed and dissected livers of Rag2$^{-/-}$;γc$^{-/-}$ mice injected with RD/12 (above) or transduced RD/12-Myog (below) cells are shown. Metastasis counts are shown in Table 4.

The results reported above have already been published (*Nanni et al., 2009*).

DISCUSSION

The metastatic spread and growth of malignant tumors from the primary site is still the most relevant problem in the field of tumor therapy. The study of metastasis biology of different tumor types will help in improving treatment outcome of patients. *In vivo* models to study the biological complexity of human tumor metastasis are based on immunodepressed mice.

In this thesis, the metastatic ability of different human sarcoma cell lines was tested in a new double knockout immunodeficient murine model, $Rag2^{-/-};\gamma c^{-/-}$ mice, compared to *nude* mice. The metastatic potential of human sarcomas was higher in $Rag2^{-/-};\gamma c^{-/-}$ mice than in *nude* mice in terms of both metastatic sites and metastasis number: in $Rag2^{-/-};\gamma c^{-/-}$ mice a strong increase of metastatic ability to lungs, liver and other sites was observed. Furthermore, the metastatic growth in $Rag2^{-/-};\gamma c^{-/-}$ mice was faster than in *nude* mice, thus allowing an earlier metastasis evaluation. The different metastatic ability shown by human tumor cells in the two murine hosts enlightens the importance of the residual host immune response and of the host-tumor interaction in the metastatic spread. $Rag2^{-/-};\gamma c^{-/-}$ mice completely lack cells of the adaptive immune system (B ad T cells), and NK cells, due to the deficiency of IL-2R γ-chain causing an impaired signalling efficiency of multiple cytokine receptors. The relative inefficiency of metastatic spread of human cell lines in *nude* mice could be attributed mainly to their NK activity, which efficiently kills circulating tumor cells thus contributing to the metastatic control in various organs (Budzynski et Radzikowski, 1994; Volpe et al, 1999; Wiltrout, 2000, Dewan et al., 2005). Routine pretreatment of *nude* mice with anti-NK antibodies, as was done here, improved metastatic spread of human tumors, but with limitations as for example the transient effect of NK depletion, and the impossibility to determine to which degree diverse parenchymatous NK populations were depleted.

Severely immunodepressed mice could provide a more favorable environment to determine tumor cell spontaneous metastatic propensity. A recent work described NOG mice as a quantitative model of pancreatic carcinoma metastasis (Suemizu et al., 2007) while in reports published in the last year, while our studies were ongoing, $Rag2^{-/-};\gamma c^{-/-}$ mice were investigated as hosts to study murine tumor and human leukemia cell behavior or to investigate molecular mechanisms that driven Ewing sarcoma growth *in vivo* (Le Devedec et al., 2009; Bertilaccio et al., 2009; Richter et al., 2009).

In this study, we showed that the combined immunodeficiency of T and B cells caused by Rag2 knockout, coupled with the NK deficit mediated by the absence of the γc interleukin receptor chain, gives rise to a superior host model for metastasis studies of human sarcomas with an impressive multi-organ metastasization both after i.v. and s.c. injection.

Besides NK control of tumor metastasization, this study enlightens another important topic related to tumor cell behavior, i.e. the presence of microenvironmental factors that could specifically influence sarcomas spread and metastasis growth in the liver.

In addition to the quantitative differences in the metastatic ability, human sarcoma cells showed a different metastatic organ-tropism in the two murine models. In particular, human sarcomas displayed a strong liver tropism in $Rag2^{-/-};\gamma c^{-/-}$ mice mainly after the experimental induction of metastases. In humans, liver tropism of sarcomas is a rare feature and mostly depends on the anatomical location of the primary tumor (Jaques et al., 1995; Pawlik et al., 2006) but recent advancement in the local tumor control led to a modified sarcoma metastatic pattern with an increase in liver secondaries (Giuliano et al., 1984). Worth of interest, the ability of visceral sarcomas to reach the liver, probably due to the absence of NK control, could be ascribed to a novel immune escape mechanism which could in turn lead to immunotherapeutic strategies to specifically potentiate local NK activity in visceral sarcomas.

On the other side, not all tested tumor cell lines gave origin to liver metastatic nodules, so that the phenomenon can be ascribed not just to the complete absence of NK cells which control circulating tumor cells. Liver tropism was the result of a tumor-specific peculiarity, because it was restricted to human sarcomas, whereas carcinomas did not colonize the liver of $Rag2^{-/-};\gamma c^{-/-}$ mice. The issue was to analyze why sarcomas and not carcinomas, which are known to metastasize the liver of cancer patients, showed this massive hepatic spread in $Rag2^{-/-};\gamma c^{-/-}$ mice. It could be hypothesized that the diverse immune components in the two murine models could affect both positively and negatively the metastatic liver-tropism. First, carcinoma cell lines could be more sensitive than sarcomas to residual immune components of $Rag2^{-/-};\gamma c^{-/-}$ mice, in particular liver Kuppfer cells that play an important role in removing tumor cells through phagocytosis and production of pro-inflammatory cytokines following tumor antigen stimulation (De Blaser et al., 1994; Philips, 1989). If this is the case, then treatments that selectively impair liver phagocytosis could enhance liver metastasis of human carcinomas (van der Bij et al., 2005). A specular hypothesis would be that $Rag2^{-/-};\gamma c^{-/-}$ mice lack immune components able to promote liver metastasization by human carcinomas, like for example IL-10 that acts as a major factor in liver metastatic propensity of colorectal carcinomas (Jessup et al., 2004). This cytokine is physiologically produced by various cell types missing in knockout $Rag2^{-/-};\gamma c^{-/-}$ mice, like activated T helper, B and NK cells. A further issue that could influence tumor-host interaction lay in the species-specificity of ligands and receptor pairs required for tumor cell adhesion, migration or proliferation. For instance the human carcinoembryonic antigen (CEA) system is evolutionarily different from the murine equivalent (Kuespert et al., 2006), and it is known that CEA expression can enhance the metastatic potential of human colorectal carcinomas as demonstrated by Jessup and colleagues (2004). To investigate these hypotheses, the viability of further “humanized” murine model transgenic

for human genes involved in cancer metastasization could be useful to produce suitable model in which to study human tumor cells biology and metastatic colonization.

The frequent but specific liver colonization and the fast metastatic growth of human sarcomas in this organ suggested the presence of microenvironmental molecular determinants acting as growth factors or chemotactic agents. Only the subset of sarcoma cells able to colonize the liver of $Rag2^{-/-};\gamma c^{-/-}$ mice showed growth and chemotactic response to HepG2 conditioned medium. The fact that liver metastatic and non-metastatic cells could be discriminated *in vitro* confirmed that the different behavior observed *in vivo* is a cell-autonomous phenotype.

The liver is the major bodily producer of IGF1 and IGF2 (Rikhof et al., 2009), and in low quantity of other factors as EGF, HGF, TGF- β, PDGF, VEGF that could express a key role in the metastatic spread (Miyamoto et al., 2005; Kmiec, 2001).

In search of molecules mediating the observed effects, IGFs appeared as a logical candidate because of sarcoma cell expression of IGF1R inducing cell proliferation and chemotaxis (Scotlandi and Picci, 2008; Rikhof et al., 2009). Chemotaxis experiments performed blocking the IGF1R axis confirmed the importance of its signalling in this experimental system. Even if the IGF1R blocking antibody significantly reduced the effect of liver supernatant, this activity was not completely abolished, thus indicating the involvement of additional mediators playing a key role, as could be expected given the complex metabolic function of the liver (Kmiec, 2001). Several studies demonstrated that the hepatocyte growth factor/scatter factor (HGF/SF) is involved in sarcoma invasive phenotype acting as a paracrine and autocrine growth and migration factor for human musculo-skeletal sarcomas (Ferracini et al., 1995; Ferracini et al., 1996). In particular, the rhabdomyosarcoma cell line

RD/18, that did show an increased growth ability in HepG2 supernatant but no migratory response, were chemoattracted by HGF (Ferracini et al., 1996).

Using immunodepressed Rag2$^{-/-}$;γc$^{-/-}$ mice it could be shown the cooperation of immune system counterpart (NK cells) and organ specific mediators (IGFs, and eventually other microenvironmental signals) in determining the metastatic potential of human sarcoma cells.

Rag2$^{-/-}$;γc$^{-/-}$ mice were successfully used in *in vivo* experiments to verify the antimetastatic ability of non-immunological approaches targeting specific factors involved in sarcoma cells metastatic propensity (IGF axis) and tumor cell differentiation ability (forced expression of Myog gene in sarcoma undifferentiated cells). The identification of IGFs as important factors of liver metastasization of human sarcomas suggested that agents specifically targeting the IGF axis or downstream signal transducers (Scotlandi and Picci, 2008) could be effective therapeutic antimetastatic approaches. The experiment of therapy of liver metastases in Rag2$^{-/-}$;γc$^{-/-}$ mice demonstrated that the dual PI3K/mTOR kinase inhibitor NVP-BEZ235 (Serra et al., 2008) strongly decreased metastatic burden to liver, lung and other metastatic sites.

Due to the high growth propensity of tumor cells, Rag2$^{-/-}$;γc$^{-/-}$ mice are a suitable model to better study the metastatic potential of RD/12 rhabdomyosarcoma cells compared to RD/12-Myogenin transduced cells. While *nude* mice did not allow a good comparison due to the low metastatic propensity of RD/12 control or treated cells, the new immunodepressed murine model clearly showed the therapeutic efficacy of the engineered differentiative behavior in RD/12 cells.

These results showed that Rag2$^{-/-}$;γc$^{-/-}$ mice are a powerful model to verify the biological and malignant characteristics of human tumor cells and to test antimetastatic targeted therapy.

SECTION 2

Rag2$^{-/-}$;γc$^{-/-}$ MICE AS A PRECLINICAL MODEL OF HUMAN ANTITUMOR IMMUNE RESPONSES

INTRODUCTION

Humanized mice models

Since the late 1980s, the development of humanized preclinical mouse models has been pursued to study the function and development of the human immune system. Humanized mice comprise normal, immunocompetent murine models transgenic for human genes (i.e., HLA or human immunoglobulins) or immunodeficient mice in which human tissues, hematopoietic stem cells (HSC) or mature peripheral-blood mononuclear cells (PBMC) have been adoptively transferred (Thomsen et al., 2005; Shultz et al., 2007). This second group of preclinical models is also defined as mouse-human chimaeras in which the function of the immune compartment is driven by human cells that proliferate, differentiate, and/or exhibit their physiological function within the murine host. Since the first reports of successful transfer of normal human peripheral blood leukocytes in *scid* mice (Bosma et al., 1983; Moseir et al., 1988) this approach has been widely applied to perform analysis of human immune function. In the last few years, new and more severely immunocompromised murine models (most notably, NOG and Rag2$^{-/-}$;γc$^{-/-}$ double knockout mice) were obtained and used in many areas of immunology, including the investigation of the ontogeny and differentiation of human immune cells, of autoimmunity mechanisms, of long-term immunological memory, of *in vivo* interactions between viruses and the immune system to define new vaccine strategies and of anticancer immunotherapies (Shultz et al., 2007, Zhang et al., 2008).

So far, evidences of human immune activation in humanized Rag2$^{-/-}$;γc$^{-/-}$ mice were obtained after stimulation with viral antigens such as Human

Immunodeficiency Virus (HIV) type 1, Epstein-Barr Virus (EBV), or Herpes Simplex Virus type 2 (Gorantla et al., 2007; Traggiai et al., 2004; Strowig et al., 2009; Kwant-Mitchell et al., 2009a) or with tetanus toxoid (Traggiai et al., 2004). The first evidences of human immune activation were obtained through the detection of specific human IgG, even if detected levels were low and not present in all treated mice.

Very few data described the application of humanized mice to study strategies to induce effective anti-tumor human immune responses. In a very recent work, Strowig and colleagues demonstrated the activation of T cell responses and interferon (IFN)-γ production in mice infected with EBV (Strowig et al., 2009). The activation of immune system in these mice was able to protect from the development of EBV-induced tumors even though, in this experimental setting, researchers were unable to detect the induction of specific anti-EBV immunoglobulins. Kwant-Mitchell and colleagues demonstrated the activation of human natural killer cells and their ability to produce IFN-γ after stimulation with human cytokines and to partially control the growth of human leukemia cells (Kwant-Mitchell et al., 2009b). These are the first promising reports of a partially functional human immune system able to protect from tumor development, but strategies to achieve a fully functional adaptive immune response to study immunological antitumor approaches are still under investigation.

Definition and source of human progenitor cells

In humans, hematopoietic stem cells (HCS) can be commonly collected from three different tissues: bone marrow, peripheral blood or umbilical cord blood. Bone marrow is the classical source of hematopoietic progenitor cells where they represent <1% of the total population. A very small number of HSC and progenitor cells circulate in the bloodstream but they can be mobilized from bone marrow after treatment with cytokines such as granulocytes-colony

stimulating factor. Blood from the umbilical cord blood is a very rich source of human progenitor cells and at present, represent a very popular source of human progenitor cells for research purposes. Other sources of HSC are represented by human fetal tissues and some examples of reconstitution with thymic or fetal liver tissues or cells purified from these sources are reported (Holyoake et al., 1999; Lan et al., 2006; Shultz et al., 2007).

Many studies of reconstitution of a human immune system were performed with hematopoietic progenitor cells sorted for CD34 positivity. CD34 is a glycosylated surface antigen that regulates hematopoietic cell adhesion to stromal cells and signal transduction of other hematopoiesis-related genes (Holyoake and Alcorn, 1994). Human $CD34^+$ progenitor cells transplanted into subletally irradiated newborn or adult NOD-*scid*, NOG or $Rag2^{-/-};\gamma c^{-/-}$ mice led to the development of human $CD45^+$ leukocytes and to differentiation of all major cell populations of the human hemato-lymphoid system was observed in transplanted mice, including dendritic cells, T cells and natural-interferon-producing cells, B cells and immunoglobulin-producing cells and to a lesser extent, NK cells (Palucka et al., 2003; Ishikawa et al., 2005; Gimeno et al., 2004).

Recent reports demonstrate the availability of human hematopoietic progenitor cells positive for the expression of the antigen CD133. The majority of $CD133^+$ progenitor cells coexpress the antigen CD34 (Bhatia, 2001, Gordon et al., 2003; Götze et al., 2007). Pioneering studies of transplantation with sorted human $CD133^+/CD34^+$ cells in NOD-*scid* mice showed a higher engraftment compared to $CD133^-/CD34^+$ cells (Handgretinger et al., 2003). In recent studies, CD133-sorted human progenitor cells, expanded *ex-vivo* through a 3 week culture with a mix of cytokines and growth factors, were analyzed for the presence of long term severe combined immunodeficient SCID-repopulating cells (SRCs). $CD133^+$ cells showed a superior frequency of SRCs and a significantly superior ability to generate progenitor cells *in vitro*

than $CD34^+$ hematopoietic cells (Suzuki et al., 2006). *In vivo*, when transplanted in NOD-*scid* or NOD-*scid* $\beta 2m^{null}$ or γc^{null} mice, $CD133^+$ progenitor cells showed a high percentage of reconstitution of human immune populations also at low cell doses (Suzuki et al., 2006, Boxall et al., 2009).

Engraftment strategies

Different transplantation strategies to establish humanized mice models have been described in which immunodepressed *scid* or NOD-*scid* mice were engrafted with human hematopoietic tissues and cells to obtain the reconstitution of a functional human immune system. Humanized mice were obtained by implanting directly mature human immune cells. The first report dated back to early 1980s, (Bosma et al., 1983; Mosier et al., 1988) when it was found that the intraperitoneal transplantation of human peripheral blood mononuclear cells (PBMC) into *scid* mice resulted in the establishment of a partially functional human immune system. High rate of reconstitution were obtained after transfer of human PBMC in severely combined immunodeficient mice as NOD-*scid* and NOD-*scid* $\beta 2M^{null}$ (Berney et al., 2001). PBMC models are mainly used in short-term analysis (no more than 4 weeks long) of human immune functions such as immunological disorders, analysis of antigen recall responses and investigation of allograft rejection. Most of the investigation in this experimental model could be limited in time due to the onset of xenogeneic graft-versus-host disease symptoms that can be monitored by weight loss in recipient mice (Pearson et al., 2008). Furthermore, the early investigated humanized models showed substantial limitations not only in the duration of reconstitution but also in the functionality of the engrafted human immune system (Manz, 2007, Legrand et al., 2006) mainly due to the leakiness of the immunodepressed murine phenotype. The intact macrophage and NK function of such murine models could control the migratory ability of PBMC through the peritoneal cavity causing low levels of human PBL engraftment.

Another approach to establish humanized mice was through engraftment of human fetal tissues such as thymus and liver in immunocompromised mice. The main advantage of this reconstitution model is that human lymphocytes develop from engrafted human progenitor cells, undergo negative selection during differentiation of human T and B lymphocytes and are tolerant to murine host antigens. Furthermore, the transplantation of fetal human thymus and liver tissue beneath the kidney capsule of immunocompromised mice resulted in the development of a well vascularized human thymus-like organ that can temporarily sustain human hematopoiesis. Human immune cells colonized both central lymphoid tissues (thymus and bone marrow) and secondary lymphoid organs (spleen and lymph nodes) (Lan et al., 2006; Joo et al., 2009; Lepus et al., 2009). A further increase in human colonization was achieved with the concomitant transplantation of liver-derived HSCs resulting in the development of a functional human immune system as demonstrated by skin xenograft rejection (Lan et al., 2006). The multi-lineage reconstitution of functional human immune T, B and NK cells in immunodeficient mice was also achieved through the injection of HSCs from different sources such as bone marrow, mobilized PBMC or umbilical cord blood (Legrand et al., 2006).

In pioneering works, the efficiency of engraftment by intraperitoneally engrafted HSCs resulted extremely age-dependent, likely due to the lower number and activity of phagocytic cells in newborn compared to adult mice (Gimeno et al., 2004). Pre-conditioning regimens were adopted to gain a long lasting and functional engraftment.

The most important achievement in engraftment ability was obtained through the injection of HSCs in irradiated mice. Sublethal irradiation schedules may differ due to the radiosensitivity of each murine model and mouse age (Pearson et al., 2008). Sublethal irradiation like treatments with chemical reagents, such as busulfan treatment of pregnant dams, could improve human cell engraftment resulting in depletion of mouse HSCs, increased concentrations of growth factors and chemoattractants and “space” for the

development and repopulation of human HSCs and immune cells in recipient mice (Robert-Richard et al., 2006; Gorantla et al., 2007). Furthermore, since liver is the main organ to contribute to perinatal hematopoiesis, and the hemato-lymphoid system undergoes a great expansion during the first weeks of life, Traggiai et al. set up a protocol of HSC engraftment which consisted in the transplantation of human hematopoietic $CD34^+$ stem cells in the liver of newborn $Rag2^{-/-};\gamma c^{-/-}$ mice which had received a sublethal dose of radiation. These mice showed human $CD45^+$ lymphocytes in main lymphopoietic organs such as thymus, bone marrow, spleen and lymph nodes and the development of human T, B, NK and dendritic cells (Traggiai 2004).

Other treatments aimed at reducing the innate immune cells in recipient mice before the injection of HSCs. In NOD-*scid* mice, treatment to reduce NK cell population were performed by administering anti-asialo GM1, anti-CD122 or anti-IL-2R βchain (TMβ-1) antibodies (Yoshino et al., 2000; Legrand et al., 2006). Macrophage depletion was achieved treating recipient mice with liposome-encapsulated dichloromethylene-biphosphonate (Rozemuller et al., 2004).

Different papers have been recently published in which comparisons in the engraftment ability of different immunodeficient murine models (Lepus et al., 2009) or of different sources of HSC (Matsumura et al., 2003; Lepus et al., 2009) were made. The variability of the reconstitution seemed to be mainly linked to the degree of immunodepression of the host murine model. NOG mice and $Rag2^{-/-};\gamma c^{-/-}$ mice were the more permissive hosts for the engraftment of human stem cells and the reconstitution of a human immune system because of their severe combined immunodepression. Studies comparing the engraftment ability of HSC from different sources in immunodeficient hosts did not showed differences in the maturation or functional ability of the engrafted human immune cells. The high variability of reconstitution observed in mice engrafted with different HSCs could be mainly linked to the number of injected cells or to

differences in the purification procedure or storage and not to the cell source (Chicha et al., 2005; Legrand et al., 2006).

AIM

The specific aim of this task was to establish a murine model of human immune response to evaluate the efficacy of immunological antitumor approaches. The aim of this work had two tasks: 1) to set up a model for a functional human immune system in $Rag2^{-/-};\gamma c^{-/-}$ mice comparing the efficiency of reconstitution by different hematopoietic progenitor cells, and 2) to immunize humanized mice with tumor cell vaccines expressing human HER-2 and to analyze anti-human HER-2 responses.

RESULTS

Reconstitution of human immune system in Rag2$^{-/-}$;γc$^{-/-}$ mice

Newborn (0-2 days old) Rag2$^{-/-}$;γc$^{-/-}$ mice were subletally irradiated and injected intrahepatically with 1-2 x 10^5 CD34$^+$ or CD133$^+$ cord blood progenitor cells. At 5-9 weeks of age, the peripheral blood leukocytes of Rag2$^{-/-}$;γc$^{-/-}$ mice were analyzed to assess the entity of the *in vivo* human repopulation. The reconstitution was evaluated as the percentage of circulating human CD45$^+$ cells on the whole circulating leukocytes. Similar levels of engraftment were observed in mice reconstituted with CD34$^+$ or CD133$^+$ progenitors, with median levels of human CD45$^+$ cells of 41% and 44%, respectively. A wide majority of mice (more than 80%) showed a percentage of circulating human CD45$^+$ cells ≥10% of all circulating leukocytes (Figure 7A). Most human leukocytes exhibited a CD19$^+$ phenotype (B cells), while CD3$^+$ cells (corresponding to T cells) were rarely observed (Figure 7B and 7C).

To determine whether B cells in recipient mice were functional, plasma samples were analyzed for the presence of human immunoglobulins. Rag2$^{-/-}$;γc$^{-/-}$ mice transplanted with both CD34$^+$ or CD133$^+$ cord blood cells showed detectable levels of human IgM with a median serum concentration of about 3.0 μg/mL (Figure 7D). A significantly different concentration of human IgG was observed ($p<0.05$, non parametric Mann Whitney test). At 5-9 weeks of age, a fraction of mice reconstituted with CD34$^+$ progenitor cells already showed IgG, while mice reconstituted with CD133$^+$ progenitors showed almost undetectable human IgG levels (Figure 7E).

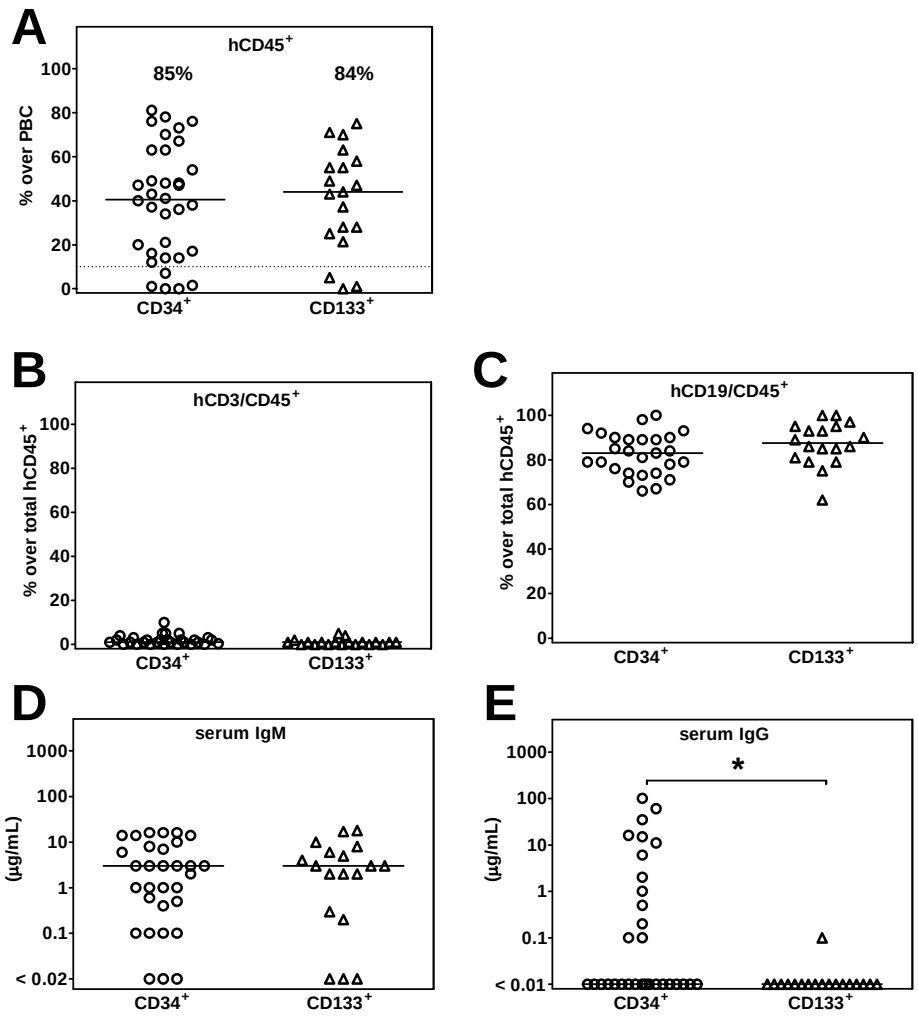

Figure 7. Reconstitution of Rag2$^{-/-}$;γc$^{-/-}$ mice with human CD34^{+} (circles) or CD133^{+} (triangles) cord blood cells: hematopoietic cell engraftment and differentiation in the peripheral blood at 5-9 weeks of age. A: overall reconstitution rate expressed as percentage of human CD45^{+} leukocytes over total number of leukocytes. **B**: percentage of human CD3^{+} cells over total human CD45^{+} leukocytes. **C**: percentage of human CD19^{+} lymphocytes over total human CD45^{+} leukocytes. **D**: serum IgM levels. **E**: serum IgG levels. * = $p<0.05$ (non parametric Mann Whitney test).

PBC: peripheral blood cells. Horizontal bars indicate median values of each series.

Kinetics of human reconstitution

The kinetics of human leukocyte populations was analyzed in a subgroup of humanized mice. The human immune reconstitution was almost stable up to about 14-16 weeks of age (Figure 8A), then a slow decrease in the percentage of circulating human $CD45^+$ leukocytes for mice engrafted with both types of human progenitor cells was observed up to 35-39 weeks of age (data not shown).

Mice engrafted with $CD34^+$ progenitors showed a significantly faster increase of the human $CD3^+$ cell population compared to mice engrafted with $CD133^+$ progenitors ($p<0.05$, Mann Whitney test at 14-16 weeks of age) (figure 8B). Rise of human $CD3^+$ population was paralleled by a decrease of human $CD19^+$, which almost disappeared from the peripheral blood at about 20-25 weeks of age (data not shown). In mice reconstituted with $CD133^+$ progenitors, $CD19^+$ lymphocytes remained high within the 14-16 weeks and significantly different from the values detected in mice reconstituted with $CD34^+$ progenitors ($p<0.05$, Mann Whitney test) (Figure 8C). On the whole, $CD19^+$ population appeared rapidly, then decreased concomitantly to $CD3^+$ rise, in agreement with data reported by Traggiai (Traggiai et al., 2004). However a slower switch between $CD19^+$ and $CD3^+$ was observed in mice reconstituted with $CD133^+$ progenitors than with $CD34^+$ progenitor cells.

Mice reconstituted with $CD34^+$ and $CD133^+$ progenitors showed a similar increase in human IgM serum levels up to 14-16 weeks after transplantation when circulating IgM reached median values of 13.5 μg/mL and 23 μg/mL, respectively (Figure 8D). Concerning IgG (Figure 8E), the difference in IgG production observed at the initial testing (see also Figure 7E) then decreased and at 14-16 weeks after reconstitution serum levels reached median values of 4.1 μg/mL versus 9.1 μg/mL, for mice engrafted with $CD34^+$ cells or $CD133^+$ cells, respectively.

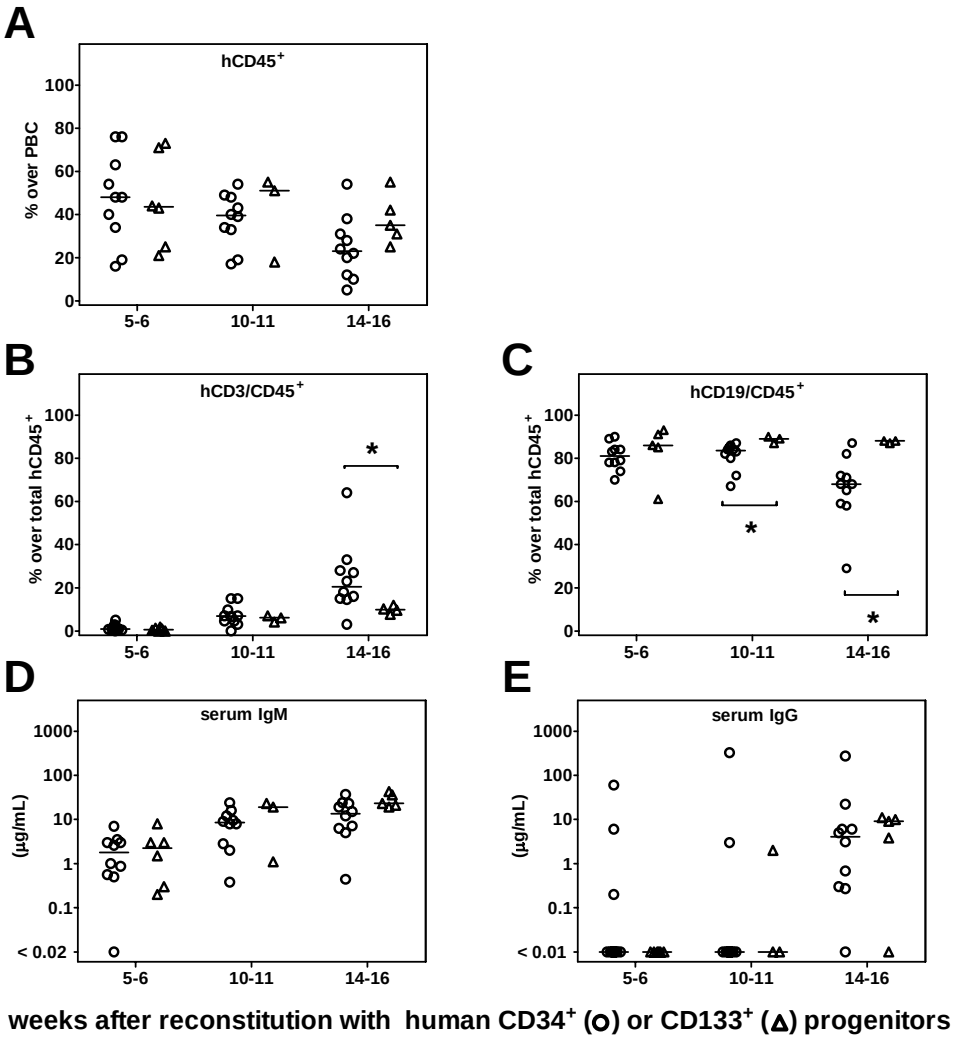

Figure 8. Kinetics of human leukocytes and immunoglobulin levels in peripheral blood of mice humanized with CD34$^+$ (circles) or CD133$^+$ (triangles) cord blood cells. **A**: percentage of human CD45$^+$ leukocytes over total number of leukocytes. **B**: percentage of human CD3$^+$ lymphocytes over total human CD45$^+$ leukocytes. **C**: percentage of human CD19$^+$ lymphocytes over total human CD45$^+$ leukocytes. **D:** serum IgM levels. **E:** serum IgG levels. * = $p<0.05$ (non parametric Mann Whitney test). PBC: peripheral blood cells. Horizontal bars indicate median values of each series.

Cell vaccination of humanized Rag2$^{-/-}$;γc$^{-/-}$ mice

To verify the functionality of the human immune system originated by CD34^{+} and CD133^{+} engrafted progenitors, and the ability to induce a specific immune response after vaccine stimulation, humanized Rag2$^{-/-}$;γc$^{-/-}$ mice with ≥10% of human leukocytes at the first test (5-6 weeks) were enrolled in protocols of cell vaccination with a cell vaccine highly positive for the expression of human HER-2 oncogene. In each reconstitution experiment, mice were randomized in two groups: vaccinated and treated mice.

The vaccination protocol consisted of two cycles of treatment based on a four-week schedule. Each cycle comprised 4 administrations scheduled in 2 weeks followed by a week of five daily administrations of human recombinant Interleukin (IL)-12 as vaccine adjuvant, and by a week of rest. Cell lines used as vaccines were a human ovary carcinoma cell line (SK-OV-3) and a murine mammary carcinoma cell line transduced with the huHER-2 gene (1E-huHER-2). After each cycle of vaccination, mice were bled to determine the level and phenotype of human lymphocytes and human immunoglobulin production to study changes in the immune parameters linked to vaccination. Since the two cell vaccines used gave similar results for each of the studied parameters, data will be presented cumulated hereafter.

In mice reconstituted with CD34^{+} progenitors (Figure 9) vaccination did not significantly modify the kinetics of human circulating CD45^{+} cells as well as that of CD3^{+} and CD19^{+} populations. Both total IgM and IgG levels were not different in control and vaccinated CD34^{+} reconstituted mice.

Mice engrafted with CD133^{+} progenitor cells also showed a similar kinetics of human CD45^{+} leukocytes in control and vaccinated groups (Figure 10A). Interestingly, vaccinated mice showed a high heterogeneity in the percentage of human CD3^{+} lymphocytes compared to control mice resulting in a slightly increased median level since 10-11 weeks after reconstitution (corresponding at the end of the first treatment cycle) ($p=0.06$) (Figure 10B).

On the other side, the median concentration of human $CD19^+$ lymphocytes in the peripheral blood of vaccinated mice was lower compared to control mice since 10-11 weeks after reconstitution ($p=0.06$) (Figure 10C). Vaccination did not modify total IgM or IgG levels (Figure 10D and 10E).

Comparison between vaccinated mice reconstituted with $CD34^+$ or $CD133^+$ progenitors showed a different kinetics of human circulating leukocytes. $CD45^+$ cells in $CD133^+$-reconstituted vaccinated mice remained significantly higher than in $CD34^+$-reconstituted vaccinated mice at the detection time of 10-11 and 14-16 weeks ($p<0.05$, non parametric Mann Whitney test). Nevertheless, the kinetics of human $CD3^+$ and $CD19^+$ lymphocyte populations were similar in vaccinated mice of the two reconstitution groups. Concerning immunoglobulin levels, $CD133^+$-reconstituted vaccinate mice showed significantly higher levels of circulating IgM than $CD34^+$-engrafted vaccinated mice 10-11 and 14-16 weeks after transplantation ($p<0.05$, non parametric Mann Whitney test). All vaccinated mice reached similar levels of circulating IgG at each detection time.

The induction of a specific anti-vaccine humoral response was analyzed with cytofluorometric assays to detect the presence of human immunoglobulins (both IgM and IgG) able to bind the human cells SK-OV-3 positive for human HER-2. More specific assays to detect human anti-HER-2 immunoglobulins were performed through ELISA or Western blot experiments. All these methods did not evidence specific anti-vaccine humoral responses in $CD34^+$- as well as in $CD133^+$-reconstituted mice (data not shown).

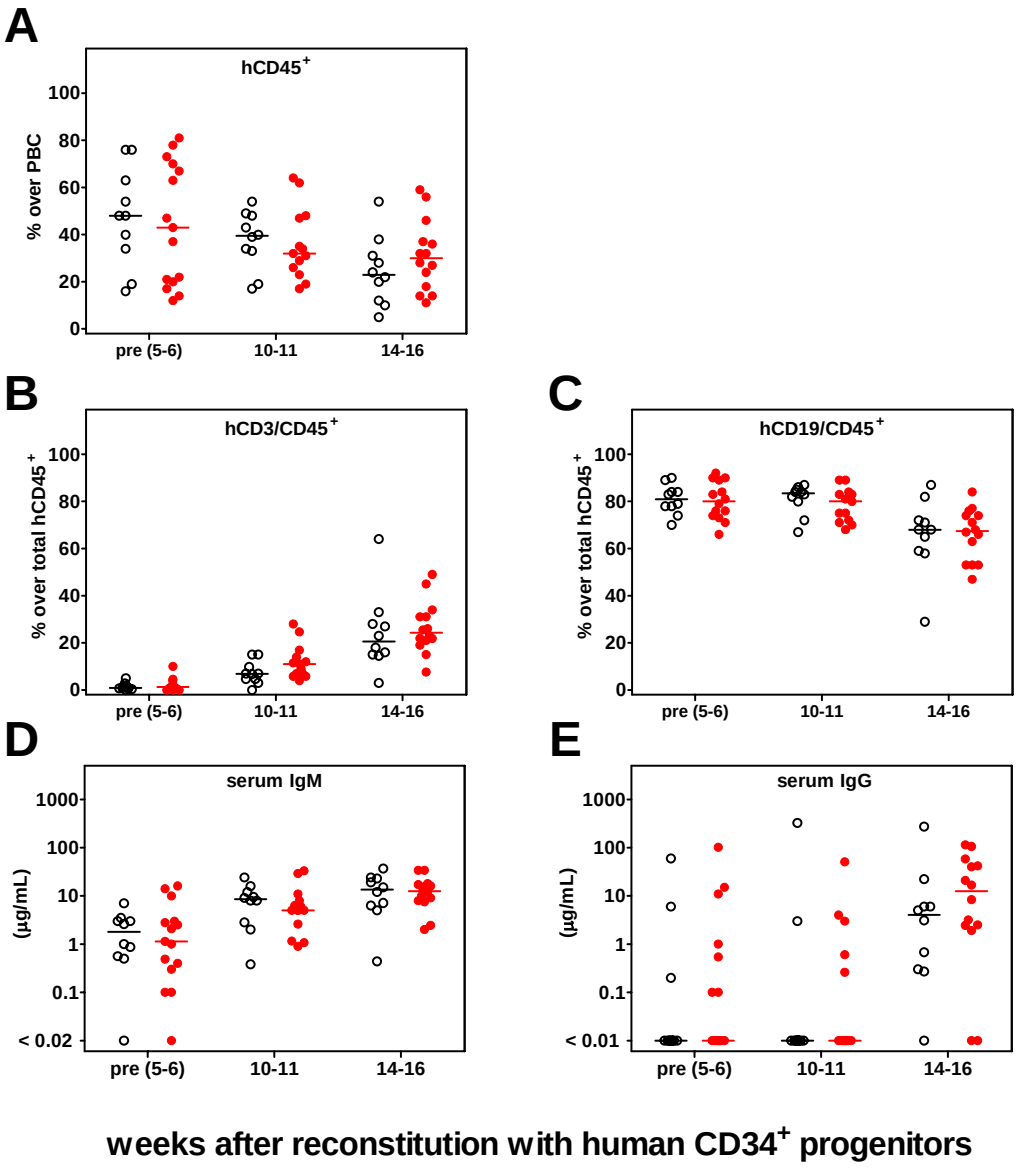

Figure 9. Kinetics of human leukocytes and immunoglobulin levels in peripheral blood of control (open circles) or vaccinated (red circles) mice reconstituted with CD34$^+$ progenitor cells. Mice were analyzed before (pre 5-6 weeks of age) and after every vaccination cycle (10-11 and 15-16 weeks after reconstitution) to detect human leukocytes and immunoglobulins in the peripheral blood. **A**: percentage of human CD45$^+$ leukocytes over total number of leukocytes. **B**: percentage of human CD3$^+$ lymphocytes over total human CD45$^+$leukocytes. **C**: percentage of human CD19$^+$ lymphocytes over total human CD45$^+$ leukocytes. **D**: serum IgM levels. **E**: serum IgG levels. PBC: peripheral blood cells. Horizontal bars indicate median values of each series.

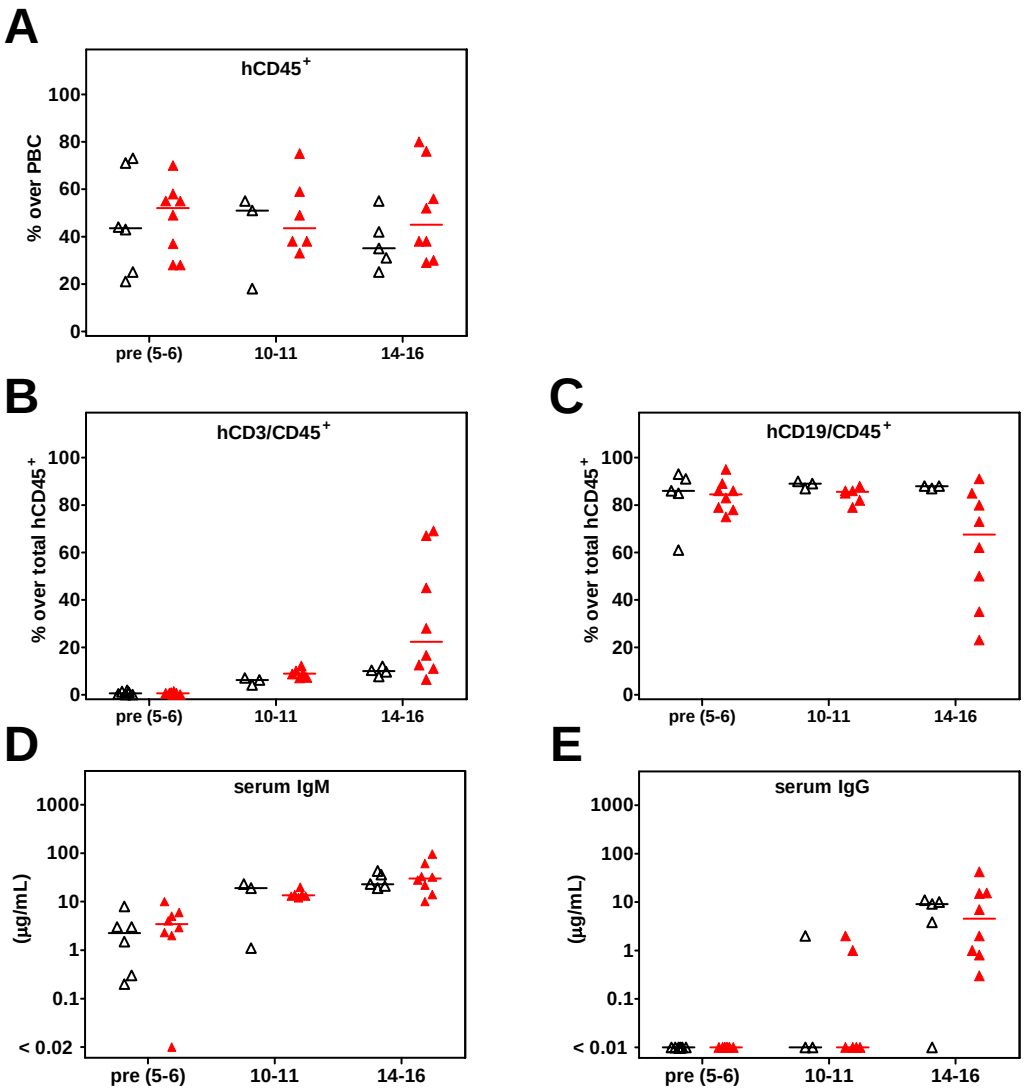

Figure 10. Kinetics of human leukocytes and immunoglobulin levels in peripheral blood of control (open triangles) or vaccinated (red triangles) mice reconstituted with CD133$^+$ progenitor cells. Mice were analyzed before (pre 5-6 weeks of age) and after every vaccination cycle (10-11 and 15-16 weeks after reconstitution) to detect human leukocytes and immunoglobulins in the peripheral blood. **A**: percentage of human CD45$^+$ leukocytes over total number of leukocytes. **B**: percentage of human CD3$^+$ lymphocytes over total human CD45$^+$leukocytes. **C**: percentage of human CD19$^+$ lymphocytes over total human CD45$^+$leukocytes. **D**: serum IgM levels. **E**: serum IgG levels. PBC: peripheral blood cells. Horizontal bars indicate median values of each series.

Localization and persistence of human leukocytes in lymphoid organs of vaccinated mice

To further compare the two progenitors used for reconstitution ($CD34^+$ and $CD133^+$), cells from several lymphoid organs (spleen, thymus, bone marrow and mesenteric lymph node) of vaccinated mice were analyzed for the presence of human cells at two different time intervals: at the end of vaccination protocol (16 weeks) and after about two further months (23 to 26 weeks of age). Human leukocytes were found in all investigated lymphoid organs up to 23-26 weeks of age (Figure 11). Similar proportions of human engraftment for mice reconstituted with the two progenitors were observed at 16 weeks of age with thymus and mesenteric lymph node almost completely reconstituted by human cells. In fact in non-reconstituted $Rag2^{-/-};\gamma c^{-/-}$ mice thymus and mesenteric lymph node are commonly not observed due to an almost complete lack of lymphoid cells (data not shown). The percentage of human $CD45^+$ leukocytes in lymphoid organs of $CD34^+$ reconstituted mice was almost stable up to 23-26 weeks, whereas for $CD133^+$ reconstituted mice a significant decrease in thymus was observed at 23-26 weeks (Figure 11).

Concerning $CD3^+$ and $CD19^+$ differentiated populations, thymus from both groups of reconstituted mice only showed $CD3^+$ cells, increasing along time. At 16 weeks of age, thymic leukocytes in mice reconstituted with both progenitors, showed a little proportion of $CD45^+$ cells showing a bright expression of human CD3, while all the other cells showed an intermediate, low or negative expression of human CD3. Furthermore, at this time, the thymus of reconstituted mice was rich in immature leukocytes double positive for the expression of human CD4 and CD8 (median levels of 79% and 72% of total leukocytes in mice reconstituted with $CD34^+$ and $CD133^+$ progenitors, respectively) that decreased along time (median levels of 67% and 24% of total leukocytes in mice reconstituted with $CD34^+$ and $CD133^+$ progenitors, respectively at 23-26 weeks of age). Spleen, bone marrow and mesenteric

lymph node contained both $CD3^+$ and $CD19^+$ populations, showing kinetics similar to those observed in the peripheral blood of untreated reconstituted mice mentioned above, with $CD3^+$ increasing and $CD19^+$ decreasing fractions along time. A more marked decrease of $CD19^+$ population was found in bone marrow of $CD133^+$ reconstituted mice at 23-26 weeks in comparison to $CD34^+$ reconstituted mice, that reached statistical significance when comparing the absolute number of $CD19^+$ cells (data not shown). Furthermore, percentage of human $CD3^+$ cells in spleen, thymus and bone marrow of $CD133^+$-reconstituted mice was significantly increased at 23-26 weeks of age compared to 16 weeks of age, while percentage of human $CD19^+$ cells in spleen and bone marrow of $CD133^+$ reconstituted mice significantly decreased along time (Figure 11).

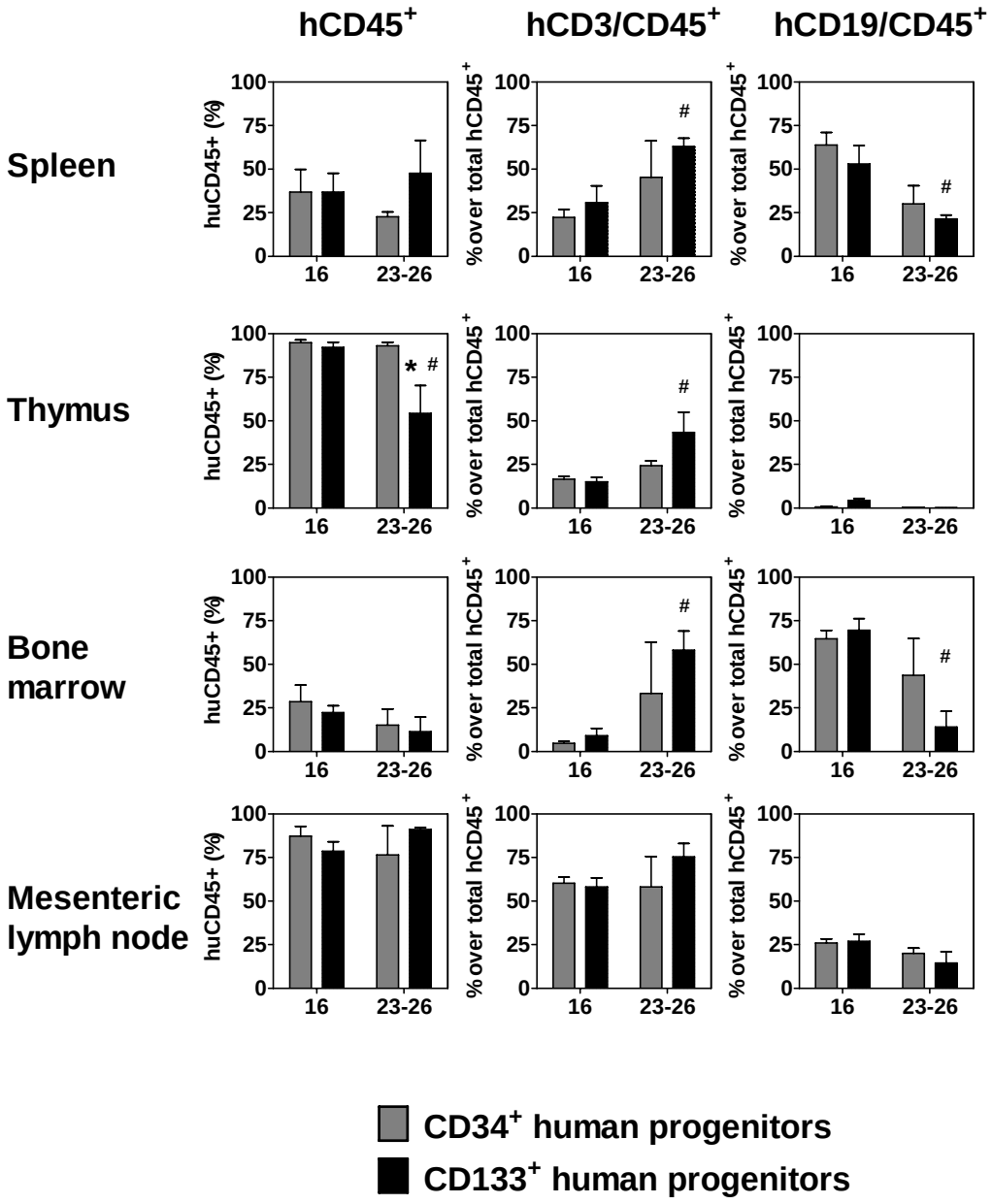

Figure 11. Human leukocytes repopulation of lymphoid organs in vaccinated mice reconstituted with $CD34^+$ (grey bars) or $CD133^+$ (black bars) progenitor cells. Mice reconstituted with $CD34^+$ or $CD133^+$ cord blood cells were sacrificed after the end of the vaccination protocol (16 weeks) or after 2 further months of observation (23-26 weeks). Mean ± SEM from 3 to 4 animals is shown for each group. * = $p<0.05$ versus mice engrafted with $CD34^+$ progenitors at the same age; # = $p<0.05$ versus 16 weeks of age (non parametric Mann Whitney test).

Tumor growth in vaccinated mice

To verify if cell vaccination was able to activate the engrafted human immune system to prevent or delay the growth of a human HER-2 positive tumor cell line (SK-OV-3), mice reconstituted in a same experimental session to reduce variability due to human cord blood cell samples and injection, were randomized into control or vaccinated group and, after the second vaccination cycle, challenged with s.c. injected SK-OV-3. Age- and sex-matched naïve (not irradiated nor engrafted with human progenitor cells) mice were challenged in parallel. As illustrated in Figure 12, reconstituted vaccinated mice showed tumor growth kinetics superimposable to that of reconstituted control mice. Humanized mice showed a reduced tumor growth rate compared to naïve mice. In this preliminary experiment, we observed that irradiated and reconstituted mice had a lower weight than naïve mice. To explain if tumor growth rate could be affected by metabolism changes due to irradiation, new experiments are ongoing to better correlate mice weight and tumor growth in naïve irradiated and reconstituted mice. On the whole, growth of SK-OV-3 cells was not modified by vaccination.

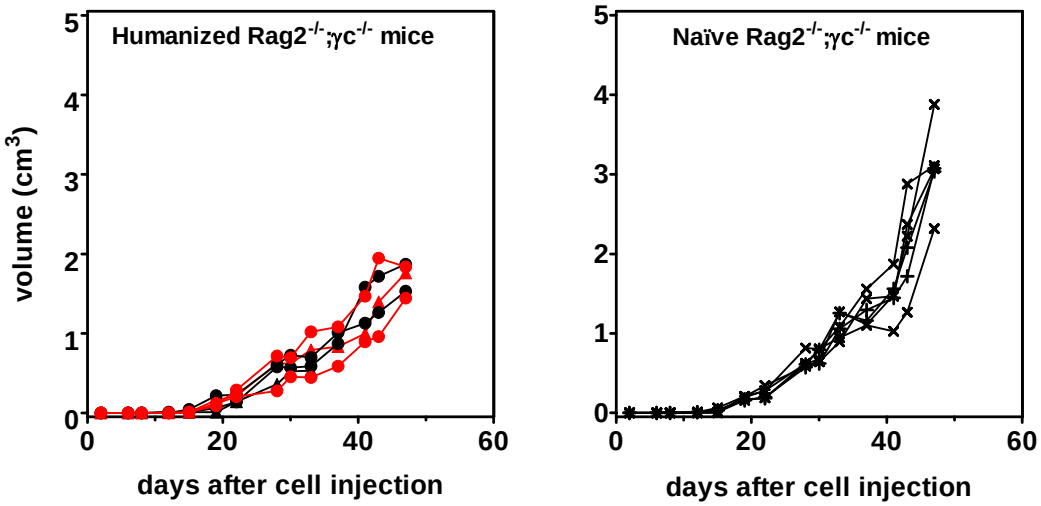

Figure 12. SK-OV-3 tumor growth in control or vaccinated Rag2$^{-/-}$;γc$^{-/-}$ mice reconstituted with CD34^{+} or CD133^{+} progenitor cells (left) and naïve Rag2$^{-/-}$;γc$^{-/-}$ mice (right). Mice received the subcutaneous injection of 10^5 SK-OV-3 cells.

Black symbols indicate control humanized mice (left panel) and naïve mice (right panel). Red symbols indicate mice vaccinated with HER-2 positive cell vaccines. Circles indicate mice engrafted with CD34^{+} progenitor cells, triangles indicate mice engrafted with CD133^{+} progenitor cells.

In vitro proliferation and IFN-γ production

To study if cell vaccination was able to induce T cell activation, control and vaccinated humanized mice were sacrificed at the end of the vaccination and human IFN-γ production by spleen cells was evaluated. Human leukocytes (enriched from spleen cell suspensions through positive magnetic sorting for human CD45 antigen) were cultured alone or in the presence of proliferation-blocked SK-OV-3 vaccine cells for six days. Then culture supernatants were collected and analyzed for the presence of human IFN-γ. IFN-γ is a cytokine released by antigen-activated T-cells and NK cells after IL-12 stimulation.

Human $CD45^+$ splenocytes from control $CD34^+$-reconstituted mice produced undetectable or very low levels of human IFN-γ after spontaneous culture, partially increased after *in vitro* restimulation with SK-OV-3 cells (Figure 13). Human $CD45^+$ spleen cells of vaccinated $CD34^+$-reconstituted mice were able to produce human IFN-γ both when cultured alone and with vaccine cells (Figure 13). Both control and vaccinated $CD133^+$ reconstituted mice showed no production of human IFN-γ by spleen cells cultured alone. Restimulation with vaccine cells did not led to significant IFN-γ production (Figure 13). In conclusion, the higher IFN-γ production found in culture of vaccinated $CD34^+$–reconstituted spleen cells with comparison to control $CD34^+$-reconstituted spleen cells suggests that some level of immune stimulation was actually obtained in these mice.

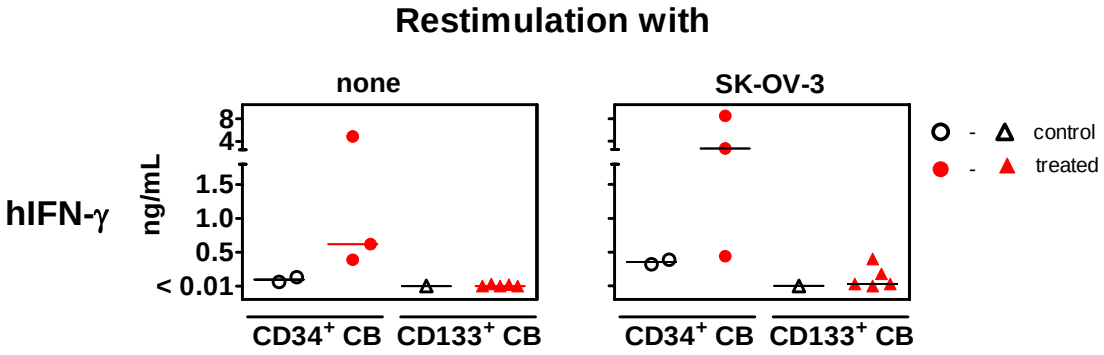

Figure 13. Human IFN-γ release by human CD45$^+$ spleen cells of mice reconstituted with CD34$^+$ (circles) or CD133$^+$ (triangles) progenitors and enrolled in cell vaccination protocols. Spleen cells were collected from age-matched control (open symbols) or vaccinated (red symbols) mice after two vaccination cycles and incubated *in vitro* for 6 days alone or with mitomycin C-treated cells expressing human HER-2 (SK-OV-3 cells). Supernatants were collected to measure the release of human IFN-γ through cytokine ELISA assays. Horizontal bars indicate median values of each series.

The results reported above will be included in a manuscript in preparation.

DISCUSSION

Humanized mice are a promising translational tool for the *in vivo* study of human hematopoiesis and immunity. The development of human adaptive immune system in immunodepressed mice reconstituted with human progenitors took advantage from the development of new more permissive immunodepressed murine models and from the definition of more efficient engraftment procedures. In 2004, Traggiai and colleagues described the development of HIS (human immune system) mice after newborn intrahepatic injection of $CD34^+$ cord blood progenitors. Because the hematolymphoid system massively expands during the first weeks of life, transplantation of stem and progenitor cells in the liver of newborn mice was able to provide a permissive environment for cell engraftment and expansion. Starting from this engraftment procedure, the work here reported was developed with two main goals: 1) to study the repopulating potential of $CD133^+$ progenitors isolated from cord blood, and 2) to evaluate the possibility to exploit $CD34^+$ or $CD133^+$ reconstituted mice to study human immune responses against HER-2 expressing tumor cells.

Irradiated $Rag2^{-/-};\gamma c^{-/-}$ newborn mice received the injection of one of two different subsets of hematopoietic progenitor cells ($CD34^+$ or $CD133^+$ cord blood cells). On the whole, about 80% of mice showed a percentage of circulating human $CD45^+$ leukocytes ≥10% of all leukocytes in short term assays (5-9 weeks), with a wide heterogeneity in $CD45^+$ levels among individual mice.

$CD133^+$ progenitors gave slightly higher levels of circulating human $CD45^+$ leukocytes from 10 to 16 weeks after transplantation maybe correlating with the content of SCID repopulating cells (SRCs) in $CD133^+$ progenitor cells (Suzuki et al., 2006). The reconstitution was stable up to 15-20 weeks of age, as data from blood samples revealed, then human leukocytes slowly decreased.

Progenitor cells showed differentiation ability in the two human major lymphoid subsets ($CD3^+$ T cells and $CD19^+$ B cells) in transplanted mice, with an early onset of circulating $CD19^+$ cells which then decrease while $CD3^+$ increase. However, some differences were detectable in mice engrafted with different progenitor cells. $CD133^+$-reconstituted mice showed a significantly higher percentage of human $CD19^+$ lymphocytes than $CD34^+$-reconstituted mice since the 10^{th} week of age. Conversely, in these mice, at 14 to 16 weeks of age, human $CD3^+$ lymphocyte percentage in the peripheral blood is significantly lower than in mice transplanted with $CD34^+$ cells.

To verify human lymphocyte activity, immunoglobulin levels were detected in serum samples of humanized mice. At the first detection time, about the 42% of mice transplanted with $CD34^+$ progenitor cells already showed detectable levels of serum IgG, whereas IgG were undetectable in almost all mice reconstituted with $CD133^+$ cells. On the other side, the two repopulating approaches showed comparable IgM levels. These data suggested that $CD133^+$ cells showed a more staminal behavior perhaps due to the major content in SRCs than $CD34^+$ sorted cells. Nevertheless, immunoglobulin concentration increased over time in the peripheral blood in all humanized mice reaching similar median levels, even though, such levels were two logs or one log lower than that measured in human adults and wild type laboratory mice, respectively (Traggiai et al., 2004; Baenziger et al., 2006).

Few studies described the induction of both humoral and cellular adaptive immune responses in $Rag2^{-/-};\gamma c^{-/-}$ humanized mice, essentially against viral pathogens (Traggiai et al., 2004; Baenziger et al., 2006; Gorantla et al., 2007, Kwant-Mitchell et al., 2009a). A future human application of anticancer cell vaccine-based strategies will require specific studies aimed at the definition, in preclinical models, of the optimal conditions for a successful translation. To study if humanized mice could be a suitable model to study human immune anti-tumor response, a standardized anti-HER-2 vaccination protocol was tested (Nanni et al., 2001; De Giovanni et al., 2004). Mice engrafted with $CD34^+$ and

$CD133^+$ cells were enrolled in protocols of cell vaccination in 4 weeks based cycles as described. No differences were detected in the kinetics of human leukocytes in the peripheral blood of vaccinated or control mice humanized with the different progenitor populations even though $CD133^+$ reconstituted vaccinated mice showed significantly higher levels of circulating $CD45^+$ leukocytes at 10-11 and 14-16 weeks after reconstitution compared to $CD34^+$ reconstituted vaccinated mice. Interestingly, in vaccinated mice engrafted with $CD133^+$ progenitors, the percentage of circulating human $CD19^+$ and $CD3^+$ cells became extremely heterogeneous compared to control mice. Namely, 14-16 weeks after reconstitution, human $CD19^+$ cells in vaccinated mice showed a reduced median percentage compared to control mice and human $CD3^+$ lymphocytes showed an increased median percentage.

As reported human B cells are functional and able to produce and release human immunoglobulins in the peripheral blood. Vaccinated mice reconstituted with each of the two progenitor cells did not show significant changes in the total concentration of human IgM or IgG compared to control mice, even though vaccinated mice reconstituted with $CD133^+$ progenitors showed significantly higher levels of human circulating IgM than vaccinated mice reconstituted with $CD34^+$ progenitors. Specific anti-vaccine antibodies were detected in none of the vaccinated mice.

All engrafted mice showed human $CD45^+$ cells in different lymphoid organs as bone marrow, spleen, thymus and mesenteric lymph node. Spleen and thymus of reconstituted animals enlarged, as reported in different papers (Traggiai et al., 2004; Chicha et al., 2005; Kwant-Mitchell et al., 2009b) and displayed quite the same size of age-matched wild-type mice. Furthermore, reconstituted mice had detectable mesenteric lymph nodes, whereas lymph nodes were never detected in naïve mice. Interestingly, comparing vaccinated mice, $CD133^+$-reconstituted mice showed a significantly reduced level of thymus engraftment from the 20^{th} to 29^{th} week of age while other lymphoid organs showed a similar or slightly superior human leukocyte persistence than

$CD34^+$-reconstituted mice. $CD3^+$ and $CD19^+$ human population efficiently differentiate in the lymphoid organs of vaccinated mice reconstituted with either progenitor cell.

The long-term loss in human $CD45^+$ cells, and mainly the absence of specific responses, were probably consequences of the absence of growth factor and cytokines that could sustain human immune leukocytes. Some murine hemato-lymphoid development relevant cytokines are not active on human immune cells (i.e. IL-2, IL-3, IL-6 and macrophage colony-stimulating factor) (Manz, 2007). The lack of cross-reactivity in cytokine function suggests that the administration and/or engineered expression of human factors able to keep human leukocyte homeostasis and induce their differentiation (Sun et al., 2006; Huntington et al., 2008; Schmidt et al., 2008; Chen et al., 2009) or the expression of human histocompatibility molecules to support human lymphocytes selection (Camacho et al., 2007) could improve human immune system maintenance.

The main actors of the immune response elicited by a similar formulation of cell vaccine were anti-$p185^{neu}$ antibodies and interferon gamma (IFN-γ) (Nanni et al., 2001; De Giovanni et al., 2004). In vaccinated humanized mice we were not able to detect the presence of human immunoglobulin specific for the oncoantigen HER-2/*neu*.

Vaccinated and control mice received the subcutaneous injection of human HER-2 positive tumor cells to test whether cell vaccination could activate human immune system to block or delay tumor growth. Humanized mice showed a reduced tumor growth rate compared to naïve mice, but no difference between control and vaccinated mice was found. Further investigations are ongoing to verify if the observed reduced tumor growth in humanized mice was due to irradiation or the consequence of some activity by human repopulating cells. Newly published data suggested that natural killer activity of engrafted human immune system could control tumor growth rate in $Rag2^{-/-};\gamma c^{-/-}$ humanized mice challenged with K562 cells (Kwant-Mitchell et al.,

2009b). Further investigations to verify the contribution of the innate immunity in our model will be performed.

Some evidence of activity by human immune cells was actually found in experiments of *in vitro* culture of human splenocytes vaccinated $CD34^{+}$-reconstituted mice: human IFN-γ production by cells from vaccinated mice (and not from controls) suggests that a T helper 1 immune stimulation was elicited by the vaccine.

All these data supported the hypothesis that the transplantation of human stem cells could allow a partially functional human immune system in which to study approaches of cancer vaccination and mimic the physiology of a human immune response. However limitations still remain like the low and quite undetectable human adaptive immune response. The generation of furtherly improved humanized models with both a decreased host innate immunity and the support of human transgenic hystocompatibility molecules and cytokines to reproduce a suitable environment for human cells maintenance and activation should therefore be pursued (Shultz et al., 2007; Zhang et al., 2008).

CONCLUSIONS

This thesis deals with the evaluation in a new preclinical murine model of two important issues for successful preclinical studies of anticancer approaches. On one side, the characterization of a suitable murine model in which to reproduce and study the physiology and the pathobiology of human cancer growth and metastasis, on the other side, the set up of a humanized model for modeling human immune antitumor responses.

The experimental studies of human tumor growth and metastasization as well as of targeted therapies required suitable immunodeficient murine models. Comparative studies reported different growth patterns in different murine models (Garofalo et al., 1993); differences were mostly attributable to the residual adaptive immune activity (i.e., granulocytes and natural killer cells) (Lozupone et al., 2000; Dewan et al., 2005). $Rag2^{-/-};\gamma c^{-/-}$ mice completely lack B, T and NK cell activity. Studies reported here showed that such severe combined immunodepression allow a better expression of the metastatic potential of human sarcomas than in *nude* mice both from a quantitatively and quantitatively point of view. Moreover, human sarcomas were able to give metastases in the liver of $Rag2^{-/-};\gamma c^{-/-}$ mice, while this organ was not affected by metastases in *nude* mice. This phenomenon could be explained as a consequence of the profound immunodepression of the experimental model (including a total absence of NK cells). Liver metastatic localization could be driven by hepatic factors acting as growth or chemoattractive factors, such as liver-produced IGFs. The potential usefulness of this potent model was suggested by the antimetastatic activity of targeted and gene-based therapeutic approaches successfully analyzed.

The development of humanized mice by engraftment with human hematopoietic stem cells could be a potential tool to study human immune system development and function. In this thesis, two different cord blood

progenitor cells were compared in terms of ability to engraft and give rise to a functional human immune system. Immune-based antitumor strategies could take advantage from the study in suitable preclinical models that allow the simulation and understanding of human immune responses. Here we showed the changes in the repopulation and differentiation of the human immune system of transplanted mice treated with tumor cell vaccines expressing human HER-2. Further analysis to detect human immune system functionality are under investigation.

MATERIALS AND METHODS

MICE

Rag2$^{-/-}$;γc$^{-/-}$ breeders were kindly provided by Drs T. Nomura and M. Ito of the Central Institute for Experimental Animals (CIEA) (Kawasaki, Japan). Mice were bred under sterile conditions in our facilities. Athymic Crl:CD-1-*Foxn1*$^{nu/nu}$ mice (here referred to as *nude* mice) were purchased from Charles River Italy and kept under sterile conditions. Experiments were authorized by the institutional review board of the University of Bologna and done according to Italian and European guidelines.

TUMOR GROWTH AND METASTATIC SPREAD OF HUMAN TUMOR CELL LINES IN RAG2$^{-/-}$;γc$^{-/-}$ MICE

Cell lines

To study the metastatic behavior of human tumor cell lines, Rag2$^{-/-}$;γc$^{-/-}$ mice received the intravenous injection of a cell suspension of sarcoma or carcinoma tumor cell lines. The panel of human sarcoma cell lines consisted in Saos-2 osteosarcoma, U2OS osteosarcoma, TC-71 and 6647 Ewing's sarcoma cell lines, gift from T. Triche (Lollini et al., 1998), SJ-Rh4 alveolar rhabdomyosarcoma (Shapiro et al., 1993) and RD/18 and RD/12, two clonal derivatives of the RD embryonal rhabdomyosarcoma cell line (Lollini et al., 1991). Human carcinoma cell lines were: HepG2 hepatocellular carcinoma (ATCC, USA), Caco-2 colo-rectal carcinoma (ATCC), HT-29 colon adenocarcinoma (ATCC), MCF7 mammary carcinoma and SK-OV-3 ovarian carcinoma, kindly provided by Dr. S. Ménard and S. Pupa, National Cancer Institute, Milan, Italy (Ricci et al., 2002). Osteosarcoma and Ewing's sarcoma

cell lines were routinely cultured in Iscove's modified Dulbecco's medium (IMDM), rhabdomyosarcoma and colon carcinoma cell lines in Dulbecco's modified Eagle's medium (DMEM), mammary and ovarian carcinoma cell lines in RPMI-1640 medium. All media were supplemented with 10% fetal bovine serum (FBS). All cells were maintained at 37°C in a humidified 5% CO_2 atmosphere with the exception of rhabdomyosarcoma cell lines, cultured in a humidified 7% CO_2 atmosphere. All medium constituents were purchased from Invitrogen (Milan, Italy).

Cell transfection

To better localize and quantify tumor metastases, TC-71 cells were transfected with pEGFP-N1 plasmid (Clontech, Mountain View, CA) carrying the *Enhanced Green Fluorescent Protein* (EGFP) gene and a neomycin resistance gene expression cassette. TC-71 cells were seeded at the concentration of $3x10^5$ cells/well in 6-well plates (Falcon, Oxnard, USA) in IMDM without serum and let to attach for 24 hours. Transfection was done with Fugene (Roche Diagnostics, Indianapolis, IN) at a 3:2 ratio (Fugene µl: DNA µg). 72 hours post transfection cells were harvested and seeded in 100 mm dishes (Nunc) in medium supplemented with geneticine (G418) (Invitrogen) (500 µg/mL). Single transfectant colonies which stably expressed EGFP were isolated using sterile glass cloning cylinders. Derived cell lines were injected in $Rag2^{-/-};\gamma c^{-/-}$ to induce experimental metastasis.

To study the role of forced expression of myogenin in rhabdomyosarcoma cell lines differentiation, RD/12 cells were transfected with a myogenin expressing plasmid. Gene transduction was done with plasmid MD-R1-Myogenin, derived from a previously described vector (Dotto et al., 1989) and kindly provided by Dr. Marco Crescenzi, Regina Elena Cancer Institute, Rome, Italy. MD-R1-Myogenin carries the 1.4 kb rat Myogenin cDNA under the control of the SV40 promoter and a neomycin resistance gene expression

cassette. Transfection was done by calcium phosphate precipitation (Invitrogen) with 2 μg of plasmid. Selection for stable transfectants was obtained through culture with DMEM + 20% fetal bovine serum containing 500 μg/mL of G418. Single transfectant colonies were isolated; control clones were isolated from cells transduced with the neomycin resistance gene alone. Myogenin expression was assessed by real-time reverse transcription-PCR.

Metastasis induction

$Rag2^{-/-};\gamma c^{-/-}$ mice (9-20 weeks-old) and *nude* mice (5-6 weeks-old) received the intravenous (i.v.) injection of viable human tumor cells (*see* Tables for cell doses) in 0.4 ml phosphate-buffered saline (PBS) (Invitrogen). 24 hours prior to the i.v. injection of human tumor cells, *nude* mice were i.v. treated with anti-asialo GM1 antiserum (Wako, Dusseldorf, Germany), 0.4 ml of a 1:30 dilution in PBS, to deplete NK activity (Kawano et al., 1986). Pilot experiments were performed to assess for each cell line at what time experimental metastases could be detected (*see* Tables).

Mice were sacrificed and subjected to an accurate necropsy. Lungs were stained with black India ink to better outline metastases and fixed in Fekete's solution. Lung and liver metastases were counted using a dissection microscope.

During the necropsy, mice who received the i.v. injection of TC-71 EGFP clones were analyzed using a fluorescence imaging device PAN-A-SEE-Y, *Panoramic Imaging System* (Lightools Research, CA, USA). To detect TC-71 EGFP metastases, filters were set up as follow: excitation filter at 470 nm wavelength, emission filter at 515 nm wavelength. Images were acquired through Qcapture software.

Some experiments were performed in which human sarcoma cells were subcutaneously (s.c.) injected (*see* Table 2 for cell doses) to study tumor growth

ability and spontaneous metastasis induction. Multi-organ metastases were evaluated as reported above.

Metastasis therapy

NVP-BEZ235 (Novartis Institutes for BioMedical Research-Oncology, Basel Switzerland) is a dual small inhibitory molecule of PI3K/mTOR kinases involved in the signalling pathway downstream IGF1R (Serra et al., 2008). NVP-BEZ235 was formulated in 5-10 N-methyl-2-pyrrolidone (NMP)/polyethylene glycol 300 (PEG300) (Fluka) (10/90, v/v) (Serra et al., 2008). Solutions (5 mg/mL) were prepared fresh each day of treatment as follows: the powder was dissolved in NMP, warmed in hot water (100°C) for 1-2 minutes, the remaining volume of PEG 300 was then added. A dose of 50 mg/kg was given *per os* daily starting from the day after cell injection. Mice received 3 drug administrations in the first week, and 5 drug administrations in the following weeks, for a total amount of 18 treatments. Mice were sacrificed 5 days after the last treatment and subjected to an accurate necropsy as described above.

Tumor cell growth and migration in conditioned media

The human liver-derived cells, HepG2 and the murine hepatic cell line, MLP29.1C (mouse liver progenitor cell line) were used to investigate *in vitro* the molecular mechanisms involved in sarcoma cell line liver metastasization. MLP29.1C cells were routinely cultured in DMEM plus 10%FBS.

To obtain conditioned medium, 4 x 10^5 human liver-derived HepG2 or MLP29.1c cells were seeded in RPMI + 10% FBS in 6-well plates. Cells were cultured for 48 h, then medium was removed, wells were washed three times with medium without serum and then serum-free DMEM or IMDM was added to each well. The incubation of the nearly confluent monolayer continued for

additional 24 hours, then conditioned medium was collected and cells conditioning the medium were harvested and counted. HepG2 conditioned medium was centrifuged at 2000 x g for 20 min at 4°C and stored at -20°C ready to be used. The same procedure was applied to aliquots of DMEM or IMDM serum-free medium to be used as control medium. For the growth assay in conditioned medium, human sarcoma and carcinoma cell lines were seeded in triplicate in 96-well plates, 10^4 cells/well, in 100 µl of HepG2 conditioned medium or control medium and cultured at 37°C in a 7% CO_2 incubator. Proliferation was evaluated after 24 and 48 h of culture, by an assay employing sulfonated tetrazolium salt WST-1 (4-[3-(4-iodo-phenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) (Roche Diagnostic, Mannheim, Germany). The measurement is based on the ability of viable cells to cleave tetrazolium salts by mitochondrial dehydrogenases. Briefly, 1/10 volume WST-1 reagent was added in each well and after 2 h incubation, absorbance of the samples was measured using a microplate reader (Sunrise TECAN, Switzerland) at 450 nm with a reference wavelength of 620 nm with control medium alone as the background level.

Migration assay was performed using Transwell chambers (Costar, Cambridge, MA) with 8 µm pore size, polyvinylpirrolidone-free polycarbonate filters. Conditioned or fresh medium was put in the lower compartments. Human tumor cultures were harvested and counted, 5 x 10^5 cells were washed and seeded in 100 µL of serum-free medium in the upper compartment of the Transwell chambers, and incubated for 18 h at 37°C in a 7% CO_2 incubator. Cells migrated through the filter to reach the lower chamber were counted using an inverted microscope or harvested and counted in a Neubauer hemocytometer. Experiments of migration in conditions of IGF1R signalling neutralization were performed by incubation of the human tumor cells with 5 µg/ml of the anti-human IGF1R monoclonal antibody clone αIR3 (Calbiochem,

Oncogene Research Products) in 100 μL of serum-free medium and then seeded in the upper compartment of the Transwell chamber.

RECONSTITUTION OF RAG2$^{-/-}$;γc$^{-/-}$ MICE WITH HUMAN HEMATOPOIETIC PRECURSORS

Newborn mice were used for experiments of reconstitution of a human immune system as previously reported (Traggiai 2004). Briefly, newborn (0-2 days old) Rag2$^{-/-}$;γc$^{-/-}$ mice were subletally irradiated with 200 cGy twice (at a 5-6 hour interval) from a Rontgen source at the Institute of Radiotherapy "L. Galvani", Dipartimento di Fisica Sanitaria (Policlinico S. Orsola Malpighi, Bologna, Italy). Radiation treatments were performed by Dott. Fabrizio Romani. Within 24 hours, mice received the intrahepatic (i.h.) injection of 1-2 x 10^5 CD34$^+$ or CD133$^+$ human cord blood progenitor cells (derived from single donors) purchased from ALLCELLS (Emerville, CA), or kindly provided by Professor Lemoli of the Department of Hematology and Oncologic Sciences "L. e A. Seragnoli", University of Bologna.

Cytofluorometric analysis of human cell engraftment

To assess the level of engraftment, at 5-9 weeks of age and subsequently every 4 weeks, mice were bled from the ventral tail vein and reconstitution was examined as circulating human CD45$^+$, CD3$^+$ and CD19$^+$ cells. Plasma samples from each mouse were collected to test human immunoglobulin levels. Cells harvested from the peripheral blood were treated with red blood cell lysis buffer. Then leukocytes were stained with PE-conjugated anti–human CD45 (clone H130) and counterstained with FITC-conjugated anti-human CD3 (clone UCHT1) or FITC-conjugated anti-human CD19 (clone HIB19) for 30 minutes on ice. Other samples were stained with PE- and FITC-conjugated mouse IgG_1, κ monoclonal immunoglobulin isotype control (clone MOPC-21). Multicolor

flow cytometric analysis of samples was performed with FACScan (Becton Dickinson, St Jose, CA). Mice with at least 10% of human $CD45^+$ cells in the peripheral blood were subjected to long-term follow up or chosen for subsequent studies. To determine human cell engraftment in lymphoid organs, mice were sacrificed at different times after engraftment and lymphoid organs (spleen, bone marrow, thymus and mesenteric lymph node) were collected and single cell suspension were analyzed for the presence of human $CD45^+$, $CD3^+$ and $CD19^+$ cells as previously described. In addition, samples from thymus were double stained with FITC-conjugated anti-human CD4 (clone RPA-T4) and PE-conjugated anti-human CD8 (clone SK1). All antibodies were purchased from BD Pharmingen (San Diego, CA).

CELL VACCINES AND TREATMENT PROTOCOL

Cell vaccines

Humanized mice were enrolled in cell vaccination protocols with human or murine cells expressing the antigen human HER-2. In combination with cell vaccine administrations, mice received the systemic injection of a potent immune adjuvant, recombinant human IL-12 (Nanni et al., 2001). The human ovary carcinoma cell line SK-OV-3 positive for human HER-2 was used in a formulation of the cell vaccine. The murine cell line used as cell vaccine, and here referred as 1E-huHER-2, was kindly provided by Dr S. Pupa (National Cancer Institute, Milan). *Neu*-negative N202.1E cells derived from a mammary carcinoma arisen in FVB-NeuN#202 mice ($H\text{-}2^q$ haplotype) transgenic for the rat *neu* proto-oncogene (Nanni et al., 2000) were transduced with human HER-2 gene to obtain 1E-huHER-2 cell line. This cell line was routinely cultured in DMEM supplemented with 20% FBS and the transgene selection maintained with G418 (400 μg/mL). During the culture passage prior to vaccination usage,

cells were cultured in medium without selection. Cells were maintained at 37°C in a humidified 5% CO_2.

Before the vaccination, SK-OV-3 and 1E-huHER-2 cells were treated with 120 and 40 μg/ml of mitomycin C (MitC; Sigma-Aldrich), respectively, to block cell proliferation. Each vaccination consisted in intraperitoneal (i.p.) injection of $2x10^6$ mitomycin C-treated cells in 0.4 mL PBS. Untreated mice received only 0.4 ml PBS.

Vaccination protocol

In humanized $Rag2^{-/-};\gamma c^{-/-}$ mice, vaccination protocols started at about 7-8 weeks of age. The vaccination protocol consisted of 4-week cycles: in the first 2 weeks, mice received four twice-weekly i.p. vaccinations followed by 1 week of five daily intraperitoneal administration of recombinant human IL-12 (PeproTech, Rocky Hill, NJ) (50 ng/mouse each administration during the first course, 100ng/mouse thereafter) in 0.2 mL of PBS supplemented with 0.01% mouse serum albumin (Sigma Aldrich, St. Louis, MO). The 4^{th} week of the cycle is a week of rest. The whole treatment consisted of two cycles of vaccination. In each reconstitution experiment, mice were randomized in two groups: vaccinated and control mice.

HER2 positive tumor cell challenge in vaccinated mice

At the end of the second cycle of treatment, vaccinated and control $Rag2^{-/-};\gamma c^{-/-}$ humanized mice received the subcutaneous injection of 10^5 SK-OV-3 HER-2^+ cells. In the same experimental session, sex- and age-matched not humanized naïve $Rag2^{-/-};\gamma c^{-/-}$ mice received the injection of SK-OV-3 cells at the same cell dose.

Tumor growth was monitored and tumor size measured twice weekly. Mice were sacrificed when tumors reached the mean volume of 2 cm^3 for

humane reasons and lymphoid organs collected, characterized phenotipically and for human IFN-γ production (see below).

IMMUNE RESPONSES

In vitro restimulation of splenocytes through mixed lymphocytes tumor cell cultures and IFN-γ release

Single cell suspensions were prepared from spleen collected from treated and control mice. Spleen cell suspensions were subjected to red blood cell lysis. Cells were washed in PBS and resuspended in RPMI 1640 supplemented with 10% FBS. Total mononuclear cells, or enriched human $CD45^+$ cells, purified by magnetic sorting (Miltenyi Biotec, Bergisch Gladbach, Germany), were cultured ($1.2x10^5$ cells/ml) for 6 days at 37°C alone or in the presence of proliferation-blocked SK-OV-3 restimulator cells ($1.2x10^4$ cells/ml) in RPMI 1640 supplemented with 10% FBS and with 50 units/ml of recombinant human IL-2. The ratio between lymphocytes and target cells was 10:1. Then culture supernatants were collected and investigated for the production of human IFN-γ by ELISA assays (Quantikine kit, R&D Systems, Abingdon, UK). ELISA assays were performed according to manufacturer instruction, the experimental range of detection of human IFN-γ was 1000 - 8 pg/mL.

Detection of human immunoglobulins and antibody response

To determine if B cells in humanized mice were functional, we assessed human antibody production by ELISA according to the manufacturer protocol (Bethyl, Montgomery TX). Mice were routinely bled from a tail vein and plasma samples collected and stored frozen at -80°C. Briefly, ELISA plates were coated overnight with anti-human IgG or IgM capture antibody. Plates were washed and blocked for 1-2 hours with a diluent solution. Standard or plasma samples, opportunely diluted, were added to the wells and incubated for

1 hour. Then plates were washed and incubated with a detection antibody conjugated to HRP. The assays were developed with tetramethylbenzidine dihydrochloride (TMB) (Thermo Scientific, Rockford, IL). The colorimetric reactions were stopped with sulphuric acid. Absorbances of samples were measured using a microplate reader (Sunrise TECAN) at 450 nm with a reference wavelength of 620 nm. Limits of detections were of 0.016 μg/ml for IgM and 0.008 μg/ml for IgG detection assays.

The production of specific antibodies induced by vaccination was studied both by ELISA assay and/or by indirect immunofluorescence assays through the binding to huHER-2 positive cells, SK-OV-3, and Western blot assays.

ELISA assay to detect antibodies recognizing human HER-2 was performed in Maxisorp NUNC 96-well microplates as reported. Plates were coated with 0.05 μg/well of huECD-HER-2 (human HER-2 extra cellular domain) overnight. After 3-4 hours blockade with assay buffer (4% w/v bovine serum albumin in PBS), wells were washed three times and incubated two hours at room temperature with plasma samples diluted (1:20 – 1:500) in assay buffer. Washed plates were then incubated with peroxidase-conjugated goat anti-human immunoglobulin G (IgG) IgA, and IgM (IgGAM) antibody (Zymed, South San Francisco, CA) for 2 additional hours at room temperature, washed again and developed with TMB. A standard curve with anti-huHER-2 Herceptin monoclonal antibody (kindly provided by Dr. S. Pupa) was run in parallel (2 - 0.008 ng/mL).

For immunofluorescent analysis of anti-human HER-2 antibodies, SK-OV-3 cells were incubated with plasma at 1:20 dilution for 30 minutes in ice, then samples were washed and incubated with a FITC conjugated anti-human IgG or anti-human IgM antibody for further 30 minutes in ice. All antibodies were purchased from BD Pharmingen. Samples were analyzed with FACScan (Becton Dickinson, St Jose, CA).

For Western Blot analysis of anti-human HER-2 antibodies, SK-OV-3 cell lisates (500 μg/sample) were immunoprecipitated with protein G PLUS-Agarose (Santa Cruz Biotechnology) after incubation with 1.5 μg of rabbit polyclonal anti-p185^{neu} serum C-18 (Santa Cruz Biotechnology, Inc) and 10 μL of plasma from vaccinated or control humanized mice. After overnight incubation, immune complexes were washed, eluted in Laemmli Sample Buffer and denaturated by heating 10 min at 100°C; then samples were resolved in 8% polyacrylamide gel. Separated proteins were electrophoretically transferred to nitrocellulose membrane (Amersham, UK) and incubated for 1 hour with rabbit polyclonal anti-p185^{neu} serum C-18 (1:250) followed by incubation with anti-rabbit Ig horseradish peroxidase-linked whole antibodies (Santa Cruz Biotechnology, Inc) 1:1000 and by a colorimetric reaction (Opti4CN Substrate kit; BioRad).

STATISTICAL ANALYSIS

All statistical analysis were performed with the software Prism (GraphPad Software, San Diego, CA, USA). A p-value of <0.05 was considered to be statistically significant.

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SCIENTIFIC PUBLICATIONS
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