Cancer Resistance as an Acquired and Inheritable Trait

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Abstract. *Aim: To induce cancer resistance in wild-type mice and detect if the resistance could be inherited to the progeny of the induced resistant mice. Furthermore to investigate the spectrum and immunology of this inherited cancer resistance. Materials and Methods: Resistance to with live S180 cancer cells in BALB/c mice was induced by immunization with inactivated S180 cancer cells. The immunization was performed by either frozen/thawed or irradiated cancer cells or cell-free ascitic fluid (CFAF). Results: In all instances the induced resistance was demonstrated to be inheritable. The phenotype was named HICR (heritable induced cancer resistance) and was defined as primary resistant progeny from mice immunized with frozen/thawed or irradiated S180 cells or CFAF obtained from mice with S180 induced ascites. Notably, this resistance was transferred from both male and female mice to the offspring of the immunized mice for at least two generations. Although inheritable, the frequency of cancer-resistant pups was lost over a few generations. Cells from the J774A.1 and RAW cancer cell lines did not induce inheritable cancer resistance, and C57BL/6 mice could not pass on cancer resistance fluorescence-activated cell sorting (FACS) analyses of the peritoneal cells revealed an increased fraction of macrophages. In necropsies of resistant mice no histological signs of cancer or other disease was found. Conclusion: Only materials derived from S180 cells could give rise to HICR mice. The molecular basis of the resistance is unknown but may involve epigenetic mechanisms. Other examples of inheritability of acquired phenotypic changes exist but, to our knowledge, this is the first demonstration of acquired, inherited cancer resistance.*

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Jean-Baptiste Lamarck (1744-1829) proposed that evolution took place through natural laws. He put forward the first coherent hypothesis attempting to explain the evolution of species, which is frequently cited for the hypothesis of inheritability of acquired traits. The traits would arise as a result of use or disuse combined with a "sentiment intérieur" and be passed on to the next generation (1). Later the notion that acquired traits to any significant extent could be trans-generationally inheritable was generally rejected. August Weisman showed in 1889 that cutting off the tail of mice in every generation for over 22 generations resulted in offspring with normal tail length, thus contributing to the rejection of the possibility of inheritance of mutilations. Weisman was the first to point-out that transmission of traits would occur *via* the germ line; effects exclusively on somatic cells would not be inheritable (2). However, in more recent times, increased recognition of the importance of epigenetics has resulted in a surge of interest in the interplay between environment and genetics and elucidation of the molecular basis of epigenetics. A number of examples of transgenerational heritability of induced phenotypic changes have been published in various species *e.g.* mice (3, 4), rats (5) and fruit flies (6, 7) and there is some epidemiological evidence for this phenomenon in humans (8, 9). A number of examples of epigenetically-based influence on ontogenic development exist. In rats, it has been demonstrated that high-fat diets to male rats affect the incidence of diabetes in their daughters, thus also showing phenotypic changes that give rise to epigenetic inheritance (5). Diethylstilboestrol may be an example of a drug inducing transgenerational epigenetic effects in humans (10).

Animal models of cancer development, as well as cancer resistance, can be useful but experience shows that caution should be exercised in extrapolating results to humans (11, 12). An interesting mouse model (the SR/CR; spontaneous regression/complete resistance mouse model) of inheritable cancer resistance to S180 carcinogenic cells was recently described (13). The origin of the cancer resistance in the founder mouse in this model was obscure and the molecular basis of resistance unknown but innate immune cells were

found to be involved. We conceived the idea that cancer resistance in some instances could be an inducible phenomenon and might be vertically transmitted. We used S180 cells to test this hypothesis. S180 cells have been used for decades as adjuvant for immunisation procedures (14, 15) and this cell line was extensively used to phenotype SR/CR mice (13). In previous studies, inactivated tumor cells have induced specific immunity in immunised mice (16-18) and in the present study we initially used frozen/thawed whole tumor cells for immunisation of the animals. The immunisation resulted in resistance to high doses of live S180 cells and we proceeded to test whether this resistance was inheritable. Immunization with irradiated S180 cells has been earlier shown to result in resistance in BALB/c mice challenged with live S180 cells (19). Except for transfer of maternal antibodies to the foetus and instances of maternal engraftment, transgenerational transfer of functional immunity against cancer is not considered to occur and has not been previously described.

Materials and Methods

Mice and cell lines. The BALB/c and C57BL/6 mice (Charles River, Sulzfeld, Germany) were either used directly or bred from breeding pairs at the Department of Experimental Medicine. All mice were housed in IVC racks (Techniplast, Varese, Italy) in ventilated polycarbonate type III cages (Techniplast). They were group-housed under standard conditions: 12 h artificial light-dark cycles, temperature was maintained at 22±2˚C and the relative humidity was 30-60%. The bedding material was Aspen chips (Tapvei, Oy, Kortteinen, Finland) and shredded cardboard and cardboard hides were used as environmental enrichment. Acidified tap water and standard rodent pellet diet (Altromin 1319, Brogaarden, Gentofte, Denmark) were provided *ad libitum*. According to the terms of the license, mice were euthanized by cervical dislocation when ascites development resulted in a weight gain of more than 5 g compared to mice of the respective strain, age and gender or when tumors exceeded the maximally allowed size of 12 mm on the longest diameter.

The S180 sarcoma cell line (American type culture collection; ATCC, Boras Sweden) was submitted to a MAP full panel testing (Taconic, Germantown, NY) and tested negative for all murine vira and mycoplasma prior to use. The J774A.1 and RAW cancer cell lines (ATCC) and the S180 cells were maintained in cell culture and transferred *in vivo,* as described below. The cells were cultured essentially as previously described (20) in Dulbecco's modified Eagle's medium (DMEM) with Glutamax-I and Hepes (Invitrogen, Taastrup, Denmark). Penicillin and streptomycin (Invitrogen) was added to a final concentration of 100 IE/ml respectively and fetal calf serum (FCS) (Invitrogen) to a final concentration of 10%. Medium was changed every second day and cells were seeded at densities of 4-5×10^5 viable cells/ml. When the cells reached a density of 1-2×10^6 viable cells/ml they were replated. In the screening procedures, the S180 cells were maintained in ascitic fluid in BALB/c mice.

In vivo cell maintenance. Initially, ascites tumor growth was induced in BALB/c mice by intraperitoneal (*i.p.*) injection of 2×10^5 S180 cells from *in vitro* culture and after about 2 weeks the mice developed ascites and were euthanized by cervical dislocation. The ascitic fluid was immediately removed by aspiration with a 10 ml syringe and 19G needle. The cells were then washed 3 times in 10 ml sterile phosphate buffered saline (PBS), centrifuged at 1250 rpm for 5 min and counted in a haemocytometer (Reichert, NY, USA) using Trypan Blue (Sigma-Aldrich, Vallensbæk, Denmark) 1:1 with the cell suspension. Cells were adjusted to 200,000 per ml in sterile PBS, kept at room temperature and used for *i.p.* injection within 1 hour. The tube containing the cells was inverted several times to keep the cells in suspension. The mice were restrained and injected *i.p.* using a 25-G needle on a 1 ml syringe and returned to their cage immediately after injection. This procedure was repeatedly used in order to maintain the S180 cells *in vivo*.

Production of founder mice. The founder BALB/c mice were produced as follows (see Figure 1 for a schematic view of the procedure): Two healthy female mice approximately eight weeks old were injected *i.p.* with 1 ml of frozen/thawed ascitic fluid containing large numbers of dead S180 cells. The frozen/thawed ascitic fluid was obtained from a BALB/c mouse that two weeks earlier had been injected with 2×10^5 S180 cells *i.p.* This mouse was euthanized and frozen in a disposable plastic bag at –20˚C for 24 h and then placed at room temperature for 4-6 h. The abdominal cavity was opened and 2 ml of the haemorrhagic ascitic fluid was aspirated into a syringe and 1 ml injected *i.p.* into each of the BALB/c female mice. Three weeks later the two mice were screened using the screening procedure described in the following section. The two founder females were mated with BALB/c males and their litters screened for the HICR (heritable induced cancer resistance) phenotype according to the screening procedure. The mice giving rise to HICR mice were termed P (for parental) mice. HICR mice could be produced using three different biological substances (i) frozen/thawed ascitic S180 cells, (ii) irradiated S180 cells or (iii) cell-free ascitic fluid (CFAF) from ascites of mice with S180 peritoneal growth. Ascitic fluid was extracted from mice immediately after euthanasia with a 10 ml syringe and a 19-G needle. The ascitic fluid was frozen in a 50 ml Falcon tube for 2 days at –80˚C and then thawed at room temperature for 4 hours. Prior to injection the cells were counted and 100% of observed cells were dead as estimated by Trypan blue dye exclusion (Sigma-Aldrich). For irradiation, ascitic fluid was collected in the same way and then gamma irradiated in a Gammacell 3000 Elan (Nordion International inc., Fleurus, Belgium) at 20 Gy in a sterile 50 ml Falcon tube. The cells were counted after irradiation and all injected mice received 1.3×10^8 irradiated S180 cells. The thawed or irradiated cells were then injected *i.p.* using a 1 ml syringe with a 25-G needle. When immunising with CFAF the ascitic fluid was obtained in the same way as described above and centrifuged at 1250 rpm for 5 min. The supernatant was isolated and frozen at –20˚C for at least two days prior to thawing at room temperature and *i.p.* injection in a 1 ml syringe with a 25-G needle. Three weeks later immunized mice were screened for the HICR phenotype as described below. None of the vaccines were in contact with other biological substances.

Screening procedure for cancer resistance. The screening procedure consisted of three consecutive injections of increasing numbers of *in vivo* maintained live S180 cancer cells and was performed as follows: At six weeks of age (females weighing approximately 16 g and males 23 g) the offspring were injected using a 25-G needle on a 1 ml syringe with 2×10^5 live S180 cells *i.p.* and weighed after the injection. After injection, the mice were observed daily and weighed two times a week and their weights were noted.

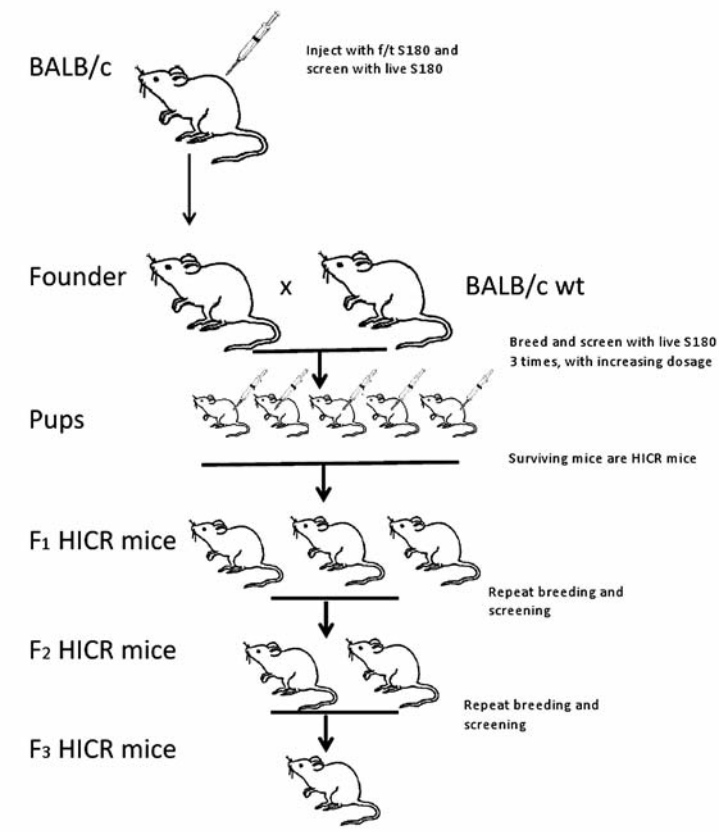

Figure 1. *A simplified depiction of the immunization and screening procedure to breed HICR mice.*

At 9 weeks of age the surviving pups were injected with 2×10^6 live S180 cells *i.p.* At 12 weeks of age the remaining mice were injected with the third and final dose of 5×10^6 live S180 cells *i.p.* Mice without ascites and normal weight gain 30 days after the third injection of live cancer cells were regarded as having the HICR phenotype. The time intervals between the screening procedure and breeding on the resistant mice were always the same.

Subcutaneous immunisation with irradiated S180 cells. Ten BALB/c mice were injected with 1×10^6 irradiated S180 cells suspended in sterile PBS with an injection volume of 0.1 ml in a 1 ml syringe with a 25-G needle in each axil region. Prior to the injections the mice were anaesthetized with a Hypnorm (10 mg/ml) (vetPharma Ltd, Leeds, UK) and Midazolam (5 mg/ml) (Hameln pharmaceuticals, Hameln, Germany) mixture with sterile water as the last component (1:1:2) in a dose of 0.1 ml/10 g body weight subcutaneously (*s.c.*). Three weeks later they were injected with 1×10^6 live S180 cells suspended in sterile PBS in each axil region in Hypnorm/Midazolam anaesthesia. Progeny from mice from these immunisations were tested for direct resistance to *s.c.* injections with live cancer cells performed as described above.

Adoptive transfer (AT) experiments. Subcutaneous tumors were induced in BALB/c mice by bilateral axillary injections with 1×10^6 S180 or RAW tumor cells with an injection volume of 0.1 ml in a 1 ml syringe with a 25-G needle. The mice were anaesthetized prior to the injections as described in the section above. When the tumors had reached a size of 4-5 mm in diameter after 6-8 days, the mice were injected *i.p.* with splenocytes and peritoneal fluid from F1 HICR (n=6) or BALB/c mice (n=3) in a 1:1 ratio, *i.e.* one donor mouse per recipient mouse. The immune cells were suspended in sterile PBS.

Two days prior to AT the donor mice were injected with 2×10^6 irradiated S180 cells *i.p.* The tumor development was observed daily and the tumor sizes measured with a caliper every second day. The tumor volume was calculated *via* the following formula: b×d×h/2 where b, d and h are the three tumor dimensions; only two dimensions were measured with the calliper and the third estimated as being similar to the shortest measured dimension.

CFAF injection in C57BL/6 mice with established tumors. S180 cell tumors were induced in nine C57BL/6 mice by *s.c.* injections of 1×10^6 S180 cells in hypnorm/midazolam anaesthesia as described above. When the tumors reached sizes of approximately 75 cm^3 the mice were injected *i.p.* with 1 ml CFAF obtained as described earlier with a 25-G needle and the tumor sizes measured every 2-3 day.

Peritoneal wash and cytospins. Forty-eight hours after *i.p.* injection of 2×10^6 S180 cells, HICR mice (n=5) were injected *i.p.* with 5 ml sterile PBS at room temperature using a 25-G needle. After euthanasia the peritoneal fluid was removed sterile with a 19-G needle and a 5 ml syringe. Cytospins were made of this peritoneal fluid and stained with Giemsa (Sigma-Aldrich).

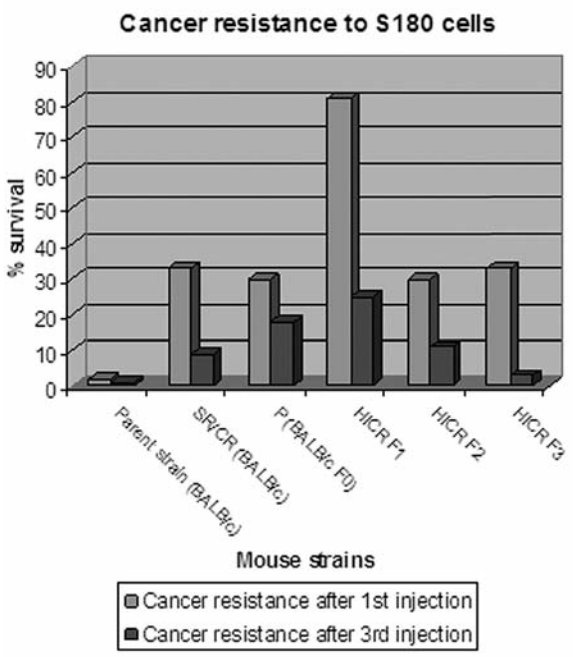

Figure 2. *Distribution of cancer resistance between the different groups of mice. There was declining survival in all tested groups of mice from 1st to 3rd injection of S180 cells. The frequency of resistance in HICR F1 mice was significantly higher compared to SR/CR mice ($p<0.0003$). The frequency of resistance in the HICR F1 mice was also significantly higher compared to BALB/c mice ($p<0.0001$). When comparing the HICR F2 mice to BALB/c mice, the frequency of resistance was also significantly higher ($p=0.0001$). In the HICR F3 mice the frequency of resistance was not significantly different from the frequency in BALB/c mice ($p=0.4774$). When comparing the frequency of cancer resistance in the SR/CR mice with BALB/c mice, the frequency in SR/CR mice was significantly higher ($p<0.0001$). All p-values by Chi-square tests. The frequencies of the resistance to the 1st S180 cell injection was in all cases higher than the resistance to the 3rd injection.*

Peritoneal fluid was centrifuged in a Cytospin2 (Shandon, Thermo scientific, Florence, Italy) at 600 rpm for 10 min at room temperature; each cuvette contained 250 μl of the peritoneal fluid. The cells were mounted on the slides which were air dried, fixated in 96% ethanol prior to Giemsa staining.

Fluorescence-activated cell sorting (FACS) analysis. The splenocytes and peritoneal fluid for the FACS analyses were harvested 48 hours after *i.p.* injection of 2×10^6 S180 cells. The leukocytes were prepared according to the manufactures' protocols and they were prior to the addition of the specific antibodies suspended in FACS media containing 10% rat serum and 10% BSA. The samples were analysed on a BD FACS Canto II flowcytometer (BD Bioscience, Mountain View, CA, USA) and the following antibodies were used: anti-CD45, anti-CD4, anti-CD19 and anti-F4/80 (BD Pharmingen, San Diego, CA, USA). The isotype controls were from Abcam (Abcam, Cambridge, UK) together with anti-CD8 and an anti-neutrophil antibody (ab53453).

The splenocytes and peritoneal cells from HICR mice (n=5) from the F_1 and F_2 generation were investigated and compared to the respective cell populations in BALB/c mice (n=5).

Statistics. The frequency of resistant progeny in the different generations was compared with parent strain mice using the Fisher's exact test and the Chi square test, respectively, depending on the group size. The FACS data of immune cell composition between BALB/c mice and the progeny from the immunized mice were tested by two-sided, unpaired Student's *t*-test. All tests were performed with a significance level of 5 % in Graph Pad Prism, 5.th edition.

License. All laboratory animal work was carried out in accordance with Danish legislation and was approved by the Animal Experiments Inspectorate (under the Danish Ministry of Justice), and all animals were inspected daily by educated animal technicians. The experimental animal work was performed at the Department of Experimental Medicine, at the University of Copenhagen, Denmark, accredited by the Association of Assessment and Accreditation of Laboratory Animal Care (AAALAC). Due to ethical considerations we strived to use as few mice as possible.

Results

Induction and inheritance of the HICR phenotype in BALB/c mice. After screening the F_1 generation (n= 30) derived from founder mice injected with frozen/thawed ascitic fluid, 26 % of the F_1 mice showed the HICR phenotype. This frequency of cancer resistance was significantly higher compared to BALB/c mice ($p<0.0001$) (Figure 2 and Table I).

In the F_2 generation the HICR phenotype was seen with a frequency of 11% which was still significantly higher compared to BALB/c mice ($p=0.0001$) (Figure 2 and Table I).

The frequency of the HICR phenotype in the F_3 generation was 3%, which was not significantly higher than the frequency in BALB/c mice ($p=0.4774$) (Figure 2 and Table I), however, in the F3 generation the number of cancer resistant mice after the first injection only was still very high compared to BALB/c mice ($p<0.0001$).

In the screening of the F_1 generation for the HICR phenotype from founders immunized with irradiated S180 cells, a single founder gave rise to offspring with the HICR phenotype that could be traced to the F_2 generation (Figure 2). In The F_3 generation one mouse resisted the first two challenges with live S180 cells but developed ascites after the third challenge. Thus, the HICR phenotype could be induced with irradiated cells but waned in the F_3 generation (Figure 3). Screening a total of 26 F_1 pups for the HICR phenotype from founders immunized with CFAF resulted in 7 HICR mice in the F_1 generation, which was significantly different from the frequency of cancer resistance in BALB/c mice ($p=0.0051$) (Figure 4A). Thus, the HICR phenotype could also be induced with CFAF (Table I).

Testing the spectrum of resistance of the HICR phenotype. Four HICR mice (two males and two females) from the F1 generation were injected with 2×10^7 S180 cells in order to

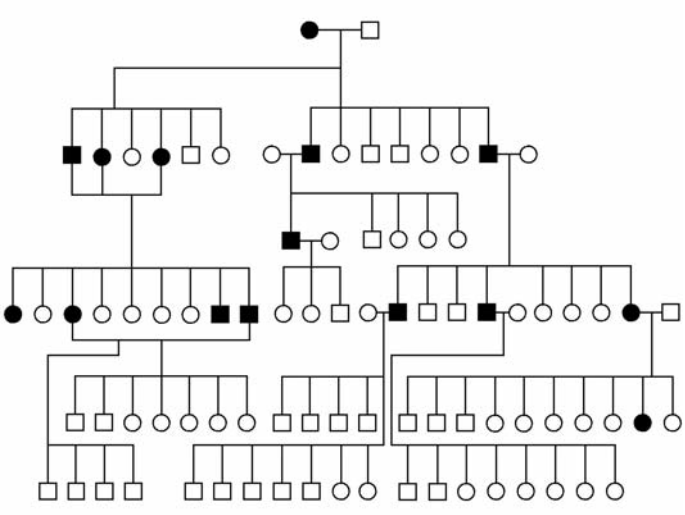

Figure 3. *Inheritance pattern of the HICR phenotype from one of the initially immunized female mice. The HICR phenotype was significantly higher compared to parent strain mice in the F1 generation (p=0.0196). The HICR phenotype was also inherited to the F2 generation with a significant higher frequency compared to BALB/c mice (p=0.0019) and was then fading to a non-significant level in the F3 generation (p=0.5059). Both female and male progeny could inherit the HICR phenotype and pass it on to their offspring.*

Table I. *Total number of cancer resistant mice of the different mouse strains/phenotypes.*

Mouse strain/phenotype	Total number of tested mice	Total number of resistant mice after first injection with S180 cells	Total number of resistant mice after third injection with S180 cells
BALB/c	200	4 (2%)	2 (1%)
SR/CR (BALB/c and B6)	391	96 (25%)	46 (12%)
P (BALB/c F0)	66	20 (30%)	18 (18%)
HICR F_1 (BALB/c)	62	50 (81%)	16 (26%)
HICR F_2 (BALB/c)	53	16 (30%)	7 (13%)
HICR F_3 (BALB/c)	52	17 (33%)	1 (2%)

test for resistance to high doses of S180 cells. Three weeks later they were injected with 10^8 S180 cells and one mouse did not develop cancer. This mouse passed the HICR phenotype on to one out of 12 pups F_1 (8%). In comparison, the overall frequency of the HICR phenotype in the F_2 generation was 13% (Table I), with these two outcomes not being significantly different (p=0.54207). Therefore, it did not seem that injection with higher doses of S180 cells could increase the frequency of inheritance of the HICR phenotype.

In order to test if the immunization route was important for obtaining the HICR phenotype, subcutaneous immunization with irradiated S180 cells was attempted. Three weeks after immunization, the mice (n=10) were injected with 1×10^6 live S180 cells in each axil region and none of the mice developed tumors. Two of the mice were bred with a male BALB/c mouse and the progeny (n=10) were at 6 weeks of age injected *s.c.* with 1×10^6 live S180 cells. Two months later 3 mice developed one *s.c.* tumor each and were euthanized; the remaining 7 mice remained tumor-free by palpation throughout the observation period of 200 days. BALB/c mice injected s.c with S180 cells have in all observed cases (n=20) been demonstrated to develop palpable solid tumors 1-2 weeks after inoculation.

Adoptive transfer of immune cells from HICR mice to BALB/c mice with RAW cell tumors showed no effect as the size of all tumors increased and reached the maximally-allowed size within 2-3 weeks after AT, whereas in histological sections no infiltration of immune cells could be recognized (Figure 4B).

Testing for inheritance of the HICR phenotype in C57BL/6 mice. Six out of eight mice resisted all three injections with S180 cells after the initial injection with frozen/thawed ascitic fluid. In total 12 pups from two of the F_0 females were tested and they all survived the 1st injection with S180 cells but they developed ascites after the 2nd injection. Thus, it seemed that the induction

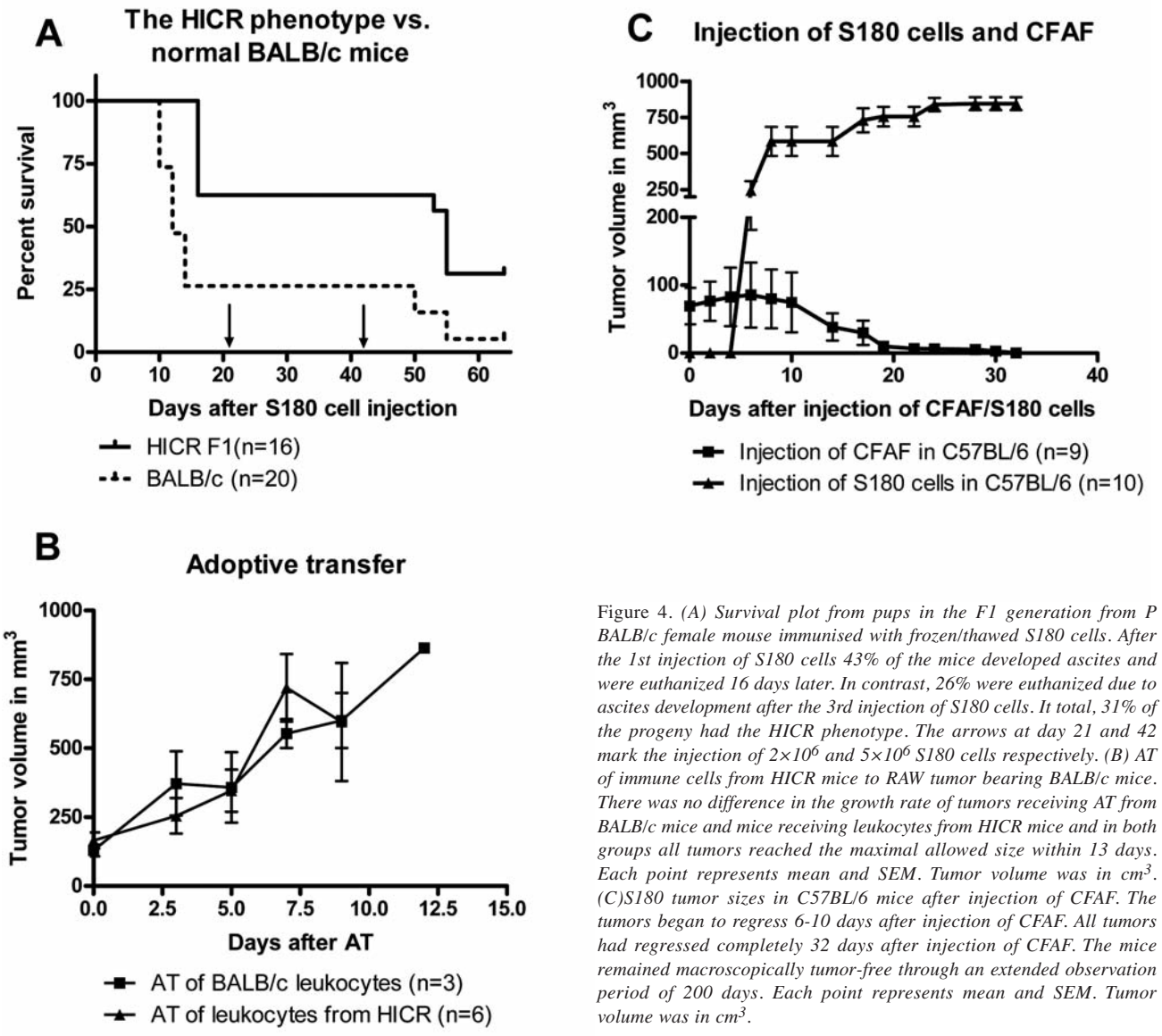

Figure 4. *(A) Survival plot from pups in the F1 generation from P BALB/c female mouse immunised with frozen/thawed S180 cells. After the 1st injection of S180 cells 43% of the mice developed ascites and were euthanized 16 days later. In contrast, 26% were euthanized due to ascites development after the 3rd injection of S180 cells. It total, 31% of the progeny had the HICR phenotype. The arrows at day 21 and 42 mark the injection of 2×10^6 and 5×10^6 S180 cells respectively. (B) AT of immune cells from HICR mice to RAW tumor bearing BALB/c mice. There was no difference in the growth rate of tumors receiving AT from BALB/c mice and mice receiving leukocytes from HICR mice and in both groups all tumors reached the maximal allowed size within 13 days. Each point represents mean and SEM. Tumor volume was in cm^3. (C)S180 tumor sizes in C57BL/6 mice after injection of CFAF. The tumors began to regress 6-10 days after injection of CFAF. All tumors had regressed completely 32 days after injection of CFAF. The mice remained macroscopically tumor-free through an extended observation period of 200 days. Each point represents mean and SEM. Tumor volume was in cm^3.*

of cancer resistance was possible in other mouse strains but the inheritance phenomenon could not be reproduced in C57BL/6 mice (Table II). CFAF was injected in nine C57BL/6 mice with established tumors. Two weeks after the injection the tumors began to regress and were macroscopically undetectable within 30 days of the CFAF injection (Figure 4C). Three weeks after complete tumor regression the mice were injected *i.p.* with 2×10^6 S180 cells. None of the mice developed ascites but in one of the male mice regrowth of the tumor was noted and this mouse was euthanized. Histologically, necrosis occupied more than 50% of the tumor area with many infiltrating PMNs (polymorphonuclear granulocytes), while no other immune cells were observed. The remaining eight mice remained macroscopically tumor-free for at least 200 days. Two of the females were bred with a C57BL/6 male. None of their 16 pups resisted the first injection with live S180 cells. When comparing with this frequency of inheritance on 0% to the HICR phenotype in the F_1 generation (Figure 1), there was a significant difference (p=0.005). This indicated that resistance to S180 cells could also be induced by immunisation with CFAF in C57BL/6 mice but not inherited to the progeny of the immunised mice in accordance with the previous finding in C57BL/6 mice immunized with frozen/thawed extract (Table II).

Peritoneal washes and FACS analyses from HICR mice 48 hours after injection of S180 cells. In the cytospins from five HICR mice from the F_1 and F_2 generation, rosette formation of immune cells in close contact with the S180 cells was

Table II. *Summary of the immunization trials in C57BL/6 mice. It was possible both with immunization with frozen/thawed S180 cells and CFAF to induce resistance to S180 cells in C57BL/6 mice but in none of the cases this resistance was demonstrated to be inheritable. The induced cancer resistance in the C57BL/6 mice was inherited in respect to resistance to the first dose of S180 cells but when increasing the dose none of the progeny had the HICR phenotype. When comparing the frequency of 0/28 mice with the HICR phenotype against the expected frequency of 26% of the HICR in BALB/c mice, this outcome is significantly different when using the Fisher's exact test (p=0.005).*

Number of mice	Immunization strategy	Survivals after 1st injection with live S180 cells	Survivals after 3rd injection with live S180 cells	Number of pups	Resistance after 1st injection	Resistance after 3rd injection
8 ♀	Thawed S180 cells in a concentration of 1.2×10^6 prior to freezing	6	6 ♀	12 (8♂+ 4♀)	7 (5 ♂ + 2♀)	0
9 (5♂+4♀)	CFAF-injected in mice with S180 cell tumors	9	8 (5♂+3♀)	16 (9♂ + 7♀)	0	0
Total=17		15/17=88%	14/17=82%	28	7/28=25%	0/28=0%

demonstrated in all cases (Figure 5). Cytospins from BALB/c mice injected with S180 cells 48 h earlier have shown large densities of S180 cells and erythrocytes (21). An increased fraction of immune cells was present in the peritoneal cavity of HICR mice 48 h after injection of S180 cells compared to BALB/c mice (Figure 6). In the BALB/c mice a large population of cancer cells was detected (Figure 6C and E), while the cancer cells were not demonstrated in the HICR mice at this time point (Figure 6B and D). The fraction of macrophages was significantly larger in HICR mice both in the spleen (p=0.0075) and in peritoneal exudate (p=0.004) compared to BALB/c mice (Figure 7A and B). In contrast, a significant larger fraction of B-cells in the splenocytes of the BALB/c compared to the HICR mice (p=0.0071) was demonstrated. The fraction of B-cells in peritoneal exudate of BALB/c mice was also larger compared to HICR mice but not to a significant level (p=0.1018). The fractions of neutrophilic granulocytes and T-cells were not significantly different between the two groups.

Discussion

Heritability of an acquired phenotype is defined as inheritance to the F_2 generation from the induced individual and, if this individual is pregnant during the induction, then the trait should be observed down to the F_3 generation (22, 23). By injection of inactivated S180 cells we can induce resistance to challenge with live S180 cancer cells. This is not surprising; however, we also showed transmission to the following generations of the phenotype and resistance to challenge with large numbers of live S180 cancer cells. We documented paternal transmission in all generations from the founder mice. In the present study, we injected biological material derived from S180 cells *i.p.* and *s.c.* and both routes of immunisation resulted in inheritable resistance to challenge with live S180 cells. This inheritable resistance was induced by the three following substances: frozen/thawed S180 cells, irradiated S180 cells and cell-free ascitic supernatant, indicating one or more soluble factors as inductors of heritable cancer resistance. The putative soluble factor(s) must impact germ line cells, thus, not crossing the Weisman barrier (2).

We failed to induce inheritable cancer resistance with J774A.1 cancer cells and AT of leukocytes from HICR mice did not kill solid RAW tumors (Figure 4B). HICR mice would inhibit S180 cells grown as solid tumors or as ascites. This resistance may be directed solely against S180 cells although we have only tested a limited number of cancer cell lines.

Presently, the only certain way to screen for the HICR phenotype is through challenge with live cancer cells and observing the outcome under standardized conditions. It is, thus, necessary to challenge the mouse itself in order to record a phenotypic trait. Survivors were monitored and randomly selected survivors bred. It can be argued that the screening procedure tests a combination of innate and adaptive immune functions in the animal as the challenge with live cancer cells is repeated 3 times with increasing dosage. When injecting control BALB/c mice with the same doses of S180 cells, the majority (96%) of the injected mice developed cancer and were subsequently euthanized (Table I). This indicates that innate mechanisms play a major part in the resistance. We have not tested if omitting the screening procedure for one generation will affect the fraction of expected HICR mice.

Early on, it was clear that BALB/c males, as well as females, were able to pass on the resistance trait to both male and female pups (Figure 3), which excludes the explanation of transplacental transmission of maternal antibodies as an explanation of the phenomenon. This was true for both primarily immunized animals, as well as animals that had inherited the cancer resistant phenotype. Inheritable cancer resistance could be induced by either frozen/thawed cancer cells, irradiated cancer cells or, more surprisingly, by CFAF and inherited by approximately 1/3 of the progeny, which at six weeks of age were shown to be directly resistant to challenge with live S180 cells. In addition, CFAF was also

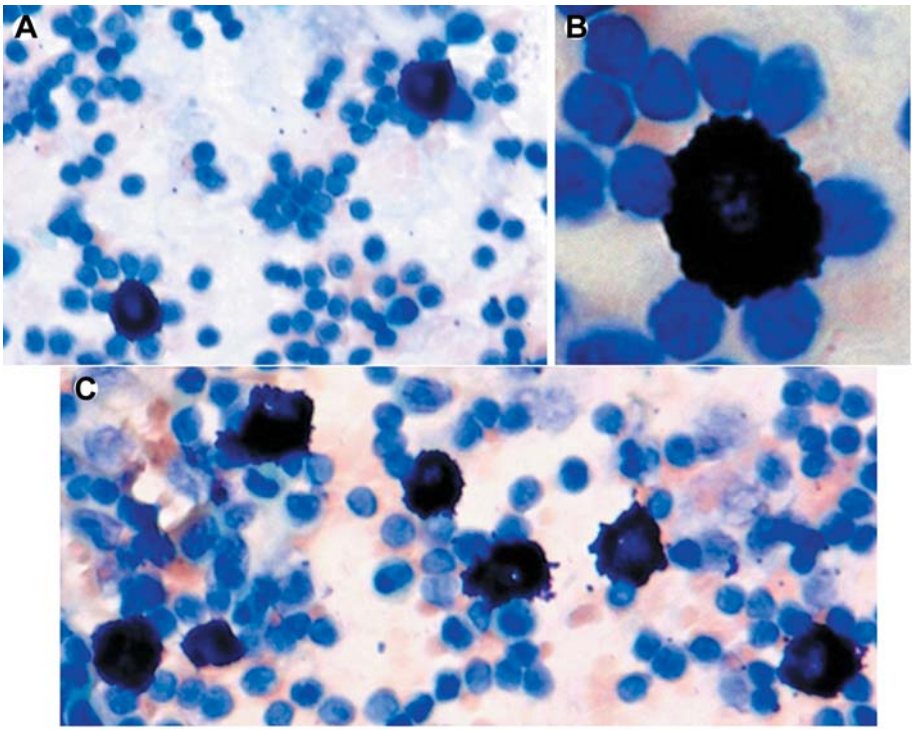

Figure 5. *Cytospins of peritoneal cells, Giemsa staining (representative pictures, n=5) (A and C 20× and B 40× magnification. Leukocytes are seen in close approximation with the S180 cells in rosette-like formations (A-C). In C, the S180 cells have disintegrated membranes indicating cell rupture.*

demonstrated to induce regression of solid S180 tumors in C57BL/6 mice (Figure 4C). The frequency of resistance decreased during three subsequent generations reaching nearly background frequency in the F_3 generation after the third challenge with live S180 cells (Figure 1 and Table I).

C57BL/6 mice did not transmit the induced resistance to their progeny (Table II), which is in accordance with other published data where it was not possible to show inheritance of an epigenetic phenotype in C57BL/6 mice, although the phenotype was inherited on a 129P4 background (24).

Taken together, the above results indicate the existence of a soluble factor(s) either produced in S180 cells or by the host as a result of interaction between S180 cells and host cells, which can induce a change in the germ line that is manifested as increased cancer resistance in the two following generations. The nature of these substances is completely unknown and it is a high priority task to isolate and purify the molecules involved in the induction of the HICR phenotype.

The phenotype of the HICR mice differs from the SR/CR phenotype (13, 25, 26) as the HICR phenotype could be repeatedly induced using biological materials from the S180 cell line and subsided over three generations. In addition, the dominant cell population after S180 *i.p.* stimulation consisted of macrophages in the HICR model. In the SR/CR mouse model of cancer resistance, the tumor destruction involved PMNs, NK (natural killer) cells and macrophages (25). These characteristics clearly distinguish the HICR mice from the SR/CR mice. If a mutation had occurred, it is highly unlikely that the same mutation(s) with similar effect could be repeatedly induced with such high frequencies with either frozen/thawed or irradiated S180 cells or CFAF (Table I). The molecular basis of the HICR phenotype is unknown, but epigenetic phenomena may, however, underlie the HICR and the SR/CR phenotypes. Other possibilities as cause of these phenotypes exist.

The rate of induction of cancer resistance in mice after immunisations with inactivated S180 cells or CFAF was not high (Figure 1 and Table I) but compared to the frequency of the SR/CR phenotype in litters from SR/CR mice (19) the rate of induction of cancer resistance was higher (p=0.0003). The resistance after the first challenge with live S180 cells continued to be high, around 30% in the F_3 generation (Table I) but, due to the nature of screening, it would be cumbersome to test for how many generations this response would remain in the HICR mice.

Others have immunized mice with irradiated S180 cells and showed that S180 cells could be inoculated together with other lethal cancer cell lines and render the mice resistant to other cancer cell lines as well. Thus, S180 cells may have the potential to work as an adjuvant in whole tumor cell vaccines (19). Allogeneic cancer cell vaccines have been

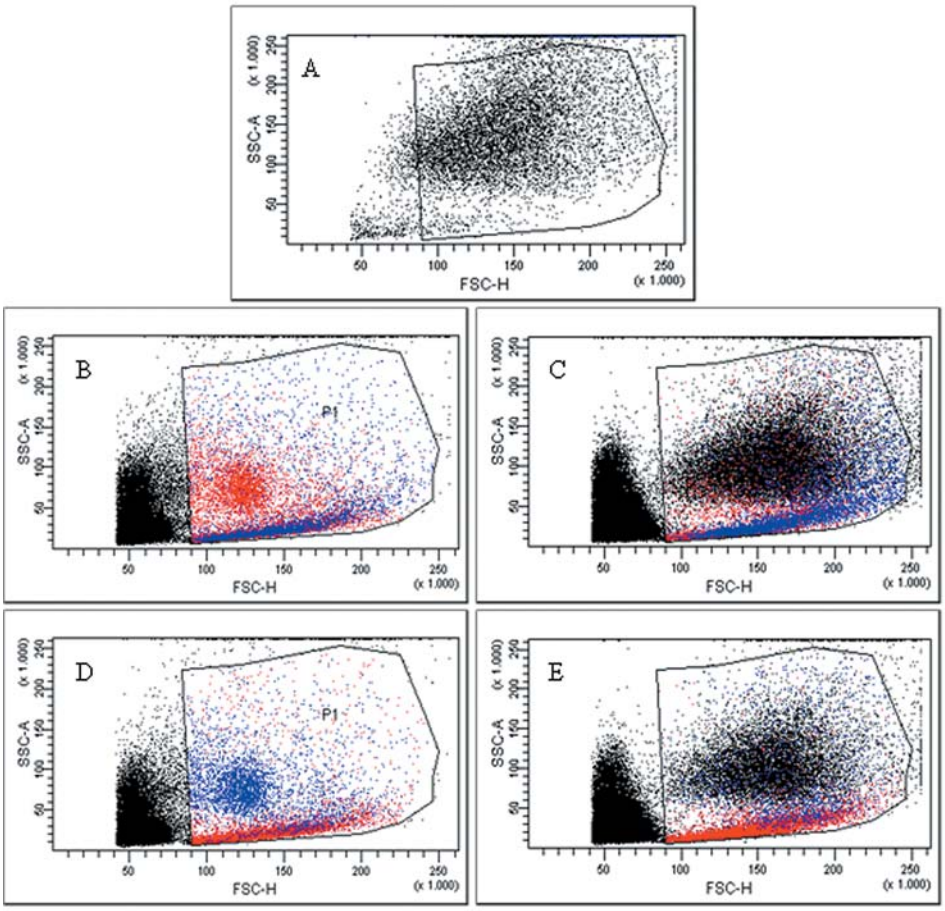

Figure 6. *FACS analyses of immune cells. (A) S180 cell culture without leukocytes and CD45+ cells. (B) Staining for CD19 (B-cells) and CD45 of peritoneal exudate cells from a HICR male mouse challenged with S180 cells 48 h earlier. This mouse was from the F2 generation from a female P mouse immunized with irradiated S180 cells. All the cells within the gate were immune cells (99% CD45+ cells) and the blue marked cells were B-cells (CD19+CD45+) and accounted for 26% of the cells in the gate. (C) Staining for CD19 (B-cells) and CD45 of BALB/c mouse challenged with S180 cells 48 h earlier. Accumulation of CD45- cells was seen and the amount of CD45+ cells was much smaller both relatively (35%). The B-cell population (blue marked cells) was also smaller (20%) but only relatively. (D) Staining for F4/80 (macrophages) and CD45 of the peritoneal exudate from the same HICR mouse as in B, F4/80+CD45+ cells (blue cells) accounted for 27%. (E) Staining for F4/80 (macrophages) and CD45 in the peritoneal exudate from same BALB/c mouse as in C, F4/80+CD45+ cells (blue cells) accounted for 6%.*

demonstrated to be more immunogenic than autologous vaccines with whole cancer cells (16). We have not tested an autologous cancer cell line.

The described findings indicate that the HICR mice have an effective immune response to S180 cells compared to normal BALB/c mice and macrophages might be involved in the killing of the cancer cells. In the FACS analyses the higher fractions of macrophages indicate that they are involved in the tumor cell destruction. We have not yet investigated the M1 and M2 phenotype of peritoneal macrophages of HICR mice (27, 28, 29).

There are a examples on induced transgenerational inheritable immunological memory: In 1980 Steele demonstrated inherited tolerance to histocompatibility antigens over two generations (30). The results we report herein are controversial and need to be independently confirmed. Our findings could pave the way for novel treatments for future human cancer therapies. Unravelling the molecular basis of the HICR-induced phenotype, as well as the substances responsible for this induction, are important future goals.

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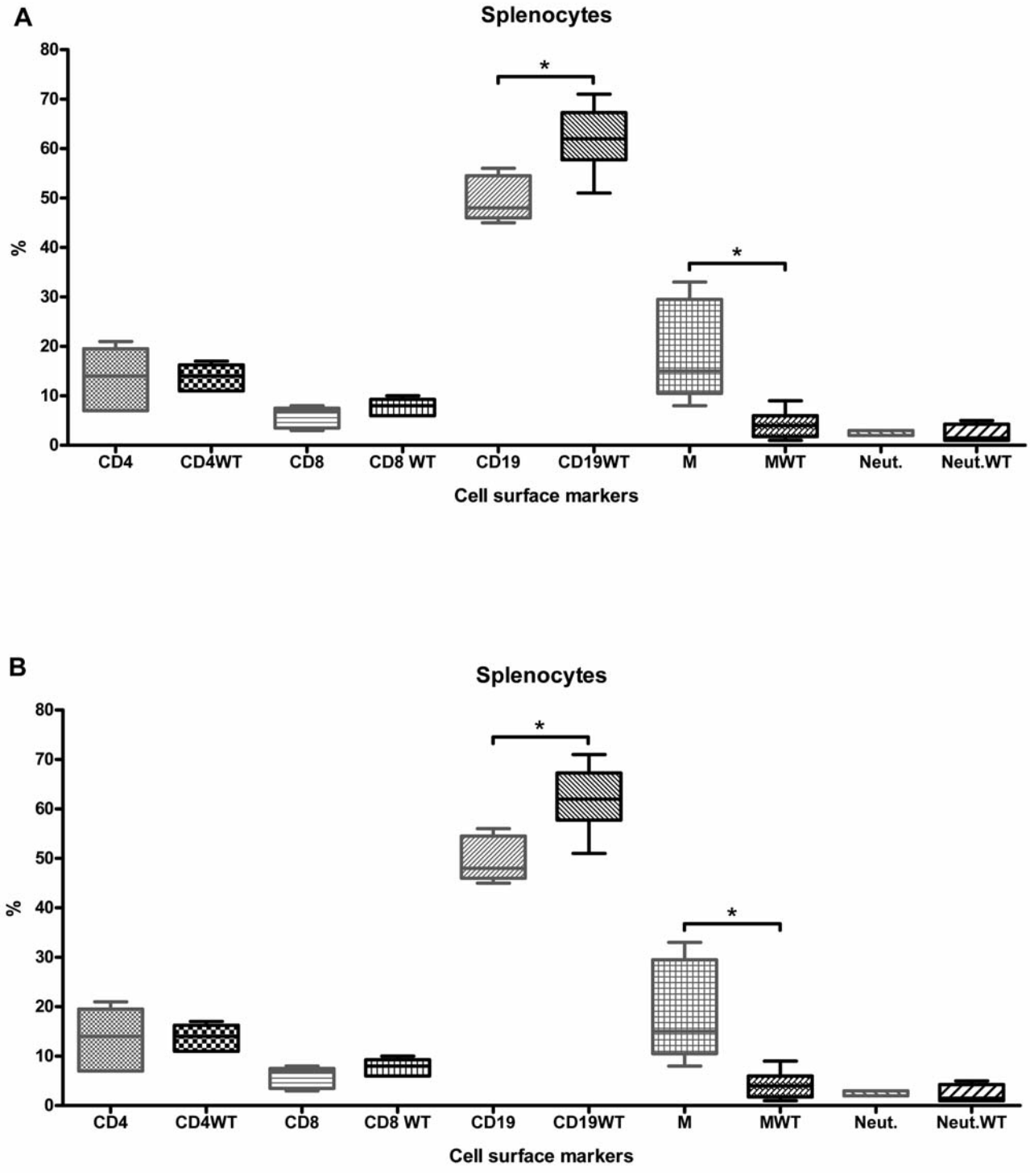

Figure 7. *Splenocytes and peritoneal cells from BALB/c mice (WT) (n=5) and HIRC mice (n=5) from the F_1 and F_2 generations from immunized mice with frozen/thawed and irradiated S180 cells. (A) Splenocytes from HICR mice and BALB/c mice. There was a significantly higher fraction of B-cells in BALB/c mice compared to HICR mice (p=0.0071). In contrast, the fraction of macrophages in HICR mice was significantly higher (p=0.0075). The other immune cells accounted for approximately the same fractions. (B) Peritoneal fluid from HICR mice and BALB/c mice. There was a significantly higher fraction of macrophages in HICR compared to BALB/c mice (p=0.0044). The other immune cells accounted for approximately the same fractions except for a non-significant higher fraction of B-cells in BALB/c mice (p=0.1018).*

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