The importance of genetic background in radiation therapy: analysis of the radiosensitivity of malignant and normal tissues

We proposed to identify genes responsible for increased radiation susceptibility. Based on the findings we were going to develop a screening test to determine normal and tumor tissue radiation sensitivity in radiotherapy patients and identify radiation sensitive individuals among radiation workers. Finally, we wanted to identify novel genes involved in radiation response and clarify their role and the involved cellular pathways.

1. The role of consensus radiation response genes in radiosensitivity

Formerly we have analyzed radiation induced gene expression patterns by whole genome microarrays in human primary fibroblast cells with different radiation sensitivity. We detected that 30 so called consensus radiation response genes answered to radiation in an identical manner in all investigated fibroblast cell lines. We selected the following genes to analyze their time- and dose dependent transcriptional responses: CDKN1A, GADD45A, IER5, CYR61, TP53INP1, GDF15 and PLK3. Cells were irradiated with different doses (10, 100, 500 mGy and 2 Gy) and total cellular RNA was isolated 2 h after irradiation. transcriptional alterations were analyzed by quantitative real time PCR. In F11 cells that present normal radiation sensitivity the expression of CYR61, CDKN1A, TP53INP1, GADD45A, PLK3 and GDF15 increased according to dose. In radiation sensitive S1 fibroblasts we have received similar results except that dose dependent response was not observed for GADD45A. To investigate time dependent transcriptional alterations cells were irradiated with 2 Gy and total RNA was isolated 1, 2, 6, 24, 48 and 72 h after irradiation. For most of the investigated genes transcription peaked 2 h after irradiation and then gradually dropped back to basal levels.

Based on the mentioned preliminary experiments we decided to investigate further the role of the GDF15 gene in radiation response and radiation sensitivity. In order to investigate the role of GDF-15 in the cellular response to ionizing radiation, cell lines in which GDF-15 expression was silenced by shRNA interference was generated. Lentiviral vectors encoding five different shRNA molecules targeting GDF-15 were transduced into F11-hTERT cells separately and from each transfection a pool of stably transduced cells (#1, #2, #3, #4 or #5) were selected from each transfection by puromycin resistance. Total RNA was isolated and used as a template to determine the level of GDF-15 suppression by quantitative real-time PCR. Two of the shRNA constructs reduced the endogenous level of GDF-15 to about 54-59% of the wild type levels and another construct to about 72%. The fourth construct did not influence significantly the level of GDF-15. Surprisingly one of the constructs enhanced the endogenous mRNA level of GDF-15 up to 2.50-fold. In a hope that we could get stronger silencing we established individual clones from the GDF-15#1 pool and also from the GDF-15#2 cell pool to study the effects of elevated GDF-15 levels. Ten-ten single colonies from shGDF-15#1 and #2 pools were picked, grown up and analyzed for GDF-15 mRNA levels. Clones F11hT-GDF15-minus and F11hT-GDF15-plus originating from shGDF-15#1 and #2 cells, respectively presented the lowest (~33%) and the highest (~242%) GDF-15 levels and was used in the experiments. Because GDF-15 is a secreted cytokine the concentration of GDF-15 was determined in the culture medium 24 and 48 h after seeding the cells by ELISA. The culture medium of wild type F11-hTERT cells contained substantial levels of GDF-15 24 h after seeding which were further increased by 48 h after plating. Barely detectable level of GDF-15 was observed in F11-hTERT-GDF-15 minus cells 24 h after seeding, although GDF-15 become detectable after 48 h. GDF-15 levels were strongly elevated in the culture medium of F11-hTERT-GDF-15 plus cells both 24 and 48 h after plating.

We investigated how the silencing of the GDF-15 gene affected its response to radiation. In wild type F11-hTERT cells the highest increase in GDF-15 expression was observed 2 h after irradiation (2.74 ± 0.8-fold increase), then GDF-15 mRNA levels decreased but still remained on an elevated level at 48 h after irradiation. In F11-hTERT-GDF-15 minus fibroblasts GDF-15 level was induced by ~1.7-fold 2 h after irradiation. It should be noted however, that even the radiation-induced levels of GDF-15 were lower than the basal level in unirradiated wild type F11-hTERT cells. Interestingly, 24 and 48 h after irradiation the GDF-15 mRNA levels decreased in F11-hTERT-GDF-15 minus cells.

In an attempt to gain insight into the cellular pathways potentially regulated by GDF-15 the basal and radiation-induced transcriptional changes of several known consensus radiation responses genes[8] such as CDKN1A (p21), GADD45A, TP53INP1 were investigated in F11-hTERT-GDF-15 minus and –plus cells. TGF-β which is related to GDF-15 was also included in the study. In unirradiated F11-hTERT-GDF-15 minus cells the basal expression levels of CDKN1A were unaltered relative to unirradiated F11-hTERT cells, while GADD45A and TGF TGF-β mRNA levels were slightly higher. A moderate down-regulation of TP53INP1 basal transcription was also observed. In GDF-15 overexpressing F11-hTERT-GDF-15 plus cells the expression of CDKN1A was unaltered. TP53INP1 mRNA expression rate increased, while TGF-β and GADD45A mRNA levels decreased. It is notable that the basal levels of the analyzed consensus radiation response genes moved in opposing directions in GDF-15 silenced and overexpressing cells.

Next we investigated radiation induced expressional changes in GDF-15-minus and –plus cells. As mentioned before, 2 Gy irradiations increased GDF-15 mRNA levels in F11-hTERT-GDF15-minus cells, but even this increased GDF-15 level was lower than the basal level in wild type F11-hTERT cells. Silencing the GDF-15 gene did not alter the radiation-response of CDKN1A. In contrast, the radiation-induced levels of TP53INP1 (2.56±0.21, 1.36±0.49, 3.195±0.18 in F11-hTERT, F11-hTERT-GDF-15 minus, F11-hTERT-GDF-15 plus) genes was lower in F11hT-GDF15-minus cells than in wild type F11-hTERT fibroblasts suggesting the involvement of GDF-15 in the radiation induced transcriptional regulation of TP53INP1.

We have investigated the involvement of GDF-15 in the radiation sensitivity of fibroblasts. Wild type, GDF-15-minus and –plus F11-hTERT cells were irradiated with 2 and 4 Gy γ-rays and the survival fractions were determined by clonogenic assay. The data clearly show that GDF-15 silenced cells are much more radiation sensitive than wild type cells. In contrast F11-hTERT-GDF15-plus cells with increased GDF-15 mRNA and secreted protein levels were slightly more radiation resistant than wild type cells.

Finally, we have studied whether silencing of the GDF-15 gene had any effect on the development of radiation-induced cell cycle blocks. First, we compared the cell proliferation rates of wild type F11-hTERT and F11hT-GDF15-minus cells. An equal number of control and GDF-15 silenced cells were plated and cells were harvested and counted at regular intervals. F11hTERT-GDF15-minus fibroblasts exhibited a similar doubling time as F11-hTERT controls (4.3 and 3.84 days, respectively), suggesting that suppression of GDF-15 did not inhibit cell proliferation *in vitro*. To investigate radiation-induced cell-cycle alterations wild type F11-hTERT and F11hT-GDF15-minus cells were irradiated with 2 Gy γ-radiations and the position of the cells within the cell cycle phases was followed by flow cytometric analysis. In irradiated F11-hTERT cells the characteristic activation of the early G2 checkpoint was clearly observable 6 h after irradiation. This block in cell cycle progression was not detectable in irradiated F11-hTERT-GDF15-minus fibroblasts, suggesting that suppression of GDF-15 abrogates the radiation-induced early G2 checkpoint. Interestingly, in F11-hTERT-GDF15 minus cells a late G2 checkpoint activation was observable at 48 h after irradiation. The late G2 block was absent in wild type cells

2. Genotyping of radiation sensitive, normal and resistant fibroblasts: single nucleotide polymorphisms and copy number variations.

To identify potential radiation sensitivity genes one can compare the genome of radiation sensitive, normal and resistant cells by analyzing single nucleotide polymorphisms (SNP) and copy number variations (CNV) with genotyping microarrays. Formerly, we have established about one hundred primary human fibroblast strains from skin samples of radiotherapy patients and determined the *in vitro* radiation sensitivity by assessing surviving fractions after 2 Gy irradiations (SF2). We found that some of the fibroblasts were radiation sensitive (SF2$\leq$15%), while others were radiation resistant (SF2$\geq$40%). To identify genes involved in radiation sensitivity and resistance we planned to genotype and compare radiation sensitive, resistant and normal fibroblasts with Affymetrix Genom-Wide Human SNP Array 6.0 in cooperation with Dr. A. Benotmane (Belgian Nuclear Research Centre, Mol, Belgium), who leads a well-equipped microarray lab perfectly suited for genotyping studies. When we started to culture the primary fibroblast cell lines for DNA isolation we realized that most of the fibroblasts stored at -75 C could not be revitalized. In these cases we isolated DNA

directly from the cells that were stored at -70. Unfortunately, it meant that these DNA isolations were unreproducible. Finally we were able to get a few μg of DNA from 12 radiation sensitive and 6 radiation resistant fibroblasts. We also isolated DNA from 22 fibroblasts showing normal radiation susceptibility and sent a total of 40 DNA samples to Dr. Benotmane for analysis. Unfortunately, the SNP analysis was not informative and in the absence of cultivable fibroblasts we were unable to repeat the experiments.

For this reason we decided to extend the scope of the project by the participation of immune cell responses in radiation sensitivity (see under point 6).

3. Differences in the basal transcriptional levels and in the radiation induced transcriptional responses of sensitive, normal and resistant fibroblasts

In order to identify genes potentially involved in radiation sensitivity we performed two sets of experiments using Agilent's whole genome microarrays. In the first set of experiments radiation responses of normal and radiation sensitive fibroblasts were investigated after irradiation with 2 Gy and genes responding only to normal and radiation sensitive cells were identified. In the second set of experiments we compared the basal transcription levels of normal and radiation sensitive primary human fibroblast cells.

Differences in the radiation response of normal and radiosensitive cells: Many genes responded to radiation either only in normal (81 up-, 112 down-regulated) or in radiation sensitive fibroblasts (116 up-, 54 down-regulated). The differentially responding genes might contribute to the radiation sensitivity of the affected strains. In normal and sensitive cells, about 20% and 17% of the annotated genes belonged to the DNA damage response, cell death and cell cycle, cell proliferation categories, respectively. The nucleotide, nucleic acid metabolism ontology group was over-represented among the annotated, up-regulated genes in sensitive cells (35%) compared to normal ones (11%). It might be important that two DNA damage response genes (MLH1, SESN1) were activated, as well as myc and IL12A were down-regulated only in normal fibroblasts. Another striking difference is that the E2F7 transcription factor (BC016658) and a mutant version (L47232) of the CDKN1/CIP1/WAF1 protein were activated by radiation in the sensitive fibroblast strains. Besides, three members (RAP2B, RRAD, BC011645) of the RAS oncogene family, as well as a cell cycle (APC2) and a programmed cell death (PDCD5) regulator protein were also up-regulated only in the sensitive fibroblasts. It might be also important that different members of the NFKB inhibitor proteins were activated in normal and sensitive strains (NFKBIα and NFKBIε, respectively).

Differences in the basal transcription profile of normal and radiation sensitive primary human fibroblast cells: Basal gene expression patterns might also influence radiation responses and susceptibility. Microarrays have been used to compare the basal gene expression patterns of normal and sensitive strains: in the first set the expression profile of radiation sensitive S1 strain was compared to the basal profile of normal cells (pooled RNA from 9 unirradiated normal cells). In the other experiment equal amounts of radiation sensitive S2 and S3 fibroblast RNAs were mixed and the combined expression pattern was compared to normal cells to get an overall pattern.

In S1 fibroblasts the basal expression of 554 genes was up- and of 734 genes were down-regulated compared to normal cells. When the combined profiles of S2 and S3 fibroblasts were analyzed, we found that 883 genes were up- and 1869 genes were down-regulated relative to normal fibroblasts. To find conserved differences and narrow down the number of genes potentially involved in radiation sensitivity the basal expression pattern of the S1 fibroblasts was compared to the profile of S2/S3 fibroblasts. The basal expression pattern of 210 genes (60 up- and 150 down-regulated) was changed in a similar manner. It suggests that these conserved alterations might be relevant in radiation sensitivity. Thirty-eight and fifty percent of the up- and down-regulated genes could be annotated to gene ontology groups, respectively. Seventeen percent of the annotated genes belong to the DNA damage response, cell cycle and proliferation, and cell death groups. The defense response (17%) and the protein metabolism (24%) categories are over-represented among the down-regulated genes. The list of potentially important genes includes DDIT4 (a DNA damage inducible protein), TP53I3 (mediates p53 responses) which were expressed at lower basal level and PDCD5, a cell death protein that was over-expressed in sensitive fibroblast strains. TP53I3 (Pig3) is induced by p53 after genotoxic stress and shares significant homology with oxidoreductases such as REDD1. It was suggested that

reduced REDD1 levels can sensitize cells towards apoptosis, whereas elevated levels of REDD1 induced for instance by hypoxia desensitize cells to apoptotic stimuli. PDCD5 is a recently identified protein that is an important regulator of both apoptotic and non-apoptotic programmed cell death.

4. Identification of radiation sensitivity biomarkers

Based on the literature data and on our preliminary experiments we realized that the analysis of the common deletions in the mitochondrial DNA (mtDNA) might be a sensitive biomarker detecting radiation responses and sensitivity. The common deletion (CD) is one of the most frequently occurring mutations in mtDNA. During the process a 4977 bp long region encoding several tRNA and respiratory chain genes is deleted from mtDNA. CD occurs after reactive oxygen species induced stress; it is related to aging processes] and age-related diseases such as Alzheimer, and atherosclerosis. Increased levels of CD are associated with Kearns-Sayre syndrome, Pearson syndrome, myopathies, chronic progressive external ophthalmoplegia, end-stage renal diseases, and certain malignant diseases including thyroid tumors, lung adenoma, colorectal- and breast cancers.

We investigated whether radiation-induced common deletions in the mitochondrial DNA follow a dose-dependent pattern and that the analysis of CD can reveal individual differences in radiation response. First, radiation sensitive immortalized S1-hTERT human fibroblasts were exposed to increasing doses of γ-radiation and total cellular DNA was isolated 72 hours later. RT-PCR was performed to measure the amount of total, wild-type (WT) and deleted (CD) mtDNA in irradiated and unirradiated control cells. The amount of total and WT mtDNA was basically unchanged, while the amount of CD increased in a dose dependent manner above 0.1 Gy. It is interesting that at low doses (10 and 50 mGy) the incidence of CD was elevated compared to higher doses, giving rise to a biphasic dose-response curve suggestive of low dose hypersensitivity in S1-hTERT fibroblasts.

Individual differences in radiation responses were investigated in two primary and two hTERT immortalized fibroblast cell lines. Based on colony forming survival assays two of the cell lines had normal radiation sensitivities (F11-hTERT and C1), whilst the other two were considered as radiation sensitive (S1-hTERT and B1). Low background levels of CD were detectable in all unirradiated cell lines by qRT-PCR. The applied SYBRGreen PCR protocol does not allow the determination of the copy numbers of total and deleted mtDNA, one can measure only their ratio. These ratios were in the range of $1.59\text{-}11.95 \times 10^{-5}$. All fibroblasts demonstrated increased CD levels 72 hours post–irradiation both at low and high doses (0.1 and 2 Gy). The radiation sensitive cell lines (B1 and S1-hTERT) showed higher CD levels at 2 Gy than normal cells (F11hTERT and C1), demonstrating that the frequency of CD reflects the individual sensitivity of the cells to higher doses of radiation.

To investigate the persistence of mtDNA deletions after irradiation normal (F11-hTERT) and radiosensitive (S1-hTERT) fibroblasts were irradiated with low and high doses (0.1 and 2 Gy). Irradiated cells were cultivated for 63 days after exposure and the incidence of total, wild type and deleted mtDNA were investigated at 7 days intervals. In 2 Gy irradiated F11-hTERT cells the CD level increased by about 1.8-folds at day 14 after IR and remained above the control level up to day 35. By day 49 after IR the amount of deleted mtDNA normalized back to control level and remained there up to 63 days. Similar pattern of CD alterations was observable after irradiating the cells with 0.1 Gy. Interestingly, the radiosensitive S1-hTERT cells demonstrated a different pattern with two waves of increased CD levels. The first CD wave was very similar to the one observed in F11-hTERT cells: the increased incidence of mtDNA deletions peaked around days 14 and then reduced back to normal level around days 35-42. However, a second increased CD level appeared by day 49 and this was still present at day 63 after IR. This increased CD level was detected both at high and low doses (2 and 0.1 Gy, respectively). We think that the first wave of elevated CD shows the long term persistence of IR induced mtDNA deletions. However, the second wave of increased CD might indicate the presence of delayed effects, namely the radiation-induced instability of the mitochondrial genome. The long term cellular consequences of the unstable mitochondrial genome are unknown. Mitochondria are the main source of energy production within the cells and they are also the main generators of free radicals, especially under pathological and stressful conditions. Recently, there has been increasing amount of evidences implicating mitochondrial dysfunction in radiation-induced bystander effects, chromosomal instability and in delayed reproductive death. Ionizing radiation may increase reactive oxygen species (ROS) levels transiently in cells, through several mechanisms including the radiolysis of water,

damaging mitochondrial DNA and/or proteins or the increased expression of inflammatory cytokines. Each of these pathways, once initiated, may perpetuate enhanced ROS and/or cytokine levels that may manifest in radiation-induced genomic instability.

5. Clinical significance of radiation sensitivity biomarkers

To investigate the clinical significance of potential radiation sensitivity marker genes (GDF15, SESN1, DDIT4, MLH1, HES1) in collaboration with the Uzsoki Hospital, Budapest we have collected tumor and normal tissues from 31 head and neck cancer patients (ETT TUKEB permission number: 132PI/2009). The tumor patients underwent surgery that was followed by radiation therapy. The biological samples were taken during surgery and stored in liquid nitrogen at the Uzsoki Hospital. Both DNA and RNA was isolated from the tumor and normal tissues at the National Institute for Radiobiology and Radiohygiene and the transcription levels, as well as the methylation status of selected marker genes were investigated. During the analysis we compared the alterations of the investigated markers in the tumor and in the corresponding normal tissues and tried to correlate it to the radiation sensitivity of the tumors and to the frequency of normal tissue sequels in the patients. The radiation susceptibility of the patients was determined by following early and late normal tissue sequel at Uzsoki Hospital.

Interestingly the expression of SESN1, HES1 and MLH1 decreased in about 70-80% of the tumor samples compared to their normal tissue counterparts. GDF15 expression increased in 5 and decreased in 3 tumor samples. Alterations in the expression of the DDIT4 gene were not informative. Interestingly, the patients with higher normal tissue GDF15 levels lived longer than those where GDF15 expression was higher in tumors, although the data are not statistically significant.

There are indications in the scientific literature that the methylation status of certain promoters might change during tumor development and it can also alter the radiation response of the corresponding genes. We have compared the methylation status of GDF15 and MLH1 genes in normal and malignant tissues. In the case of GDF15 the promoter was methylated both in normal and tumor tissues. In the case of MLH1 promoter we found that neither the tumor nor the normal tissue derived promoter was methylated. In two cases both the normal tissue and the tumor derived promoter was methylated. In 4 samples only the tumor derived promoter was methylated. So far, MLH1 promoter methylation was not related to increased patient survival.

In three of the radiation therapy treated patient early normal tissue reactions were detected. So far late sequel was absent. The follow up time of the patients is not sufficiently long to draw conclusions between marker gene alterations and normal tissue reactions.

6. Importance of the immune system in the in vivo radiation response

The normally functioning immune system is inevitable for the control of neoplastic growth; therefore, radiation-induced immune system alterations will have a strong effect on the outcome of radiotherapy. Therefore we have investigated the effects of low (0.1 Gy) and high-dose (2 Gy) irradiations on several immune parameters in experimental animals. Low dose irradiation might mimic the effect of diagnostic irradiation, such as CT scans on the immune system, while 2 Gy irradiations are usually applied as the fraction dose during radiotherapy. Animals were subjected to total-body irradiation; splenocytes were isolated four hours thereafter and assessed immediately for apoptosis. Spontaneous apoptosis of unfractionated splenocytes was 3.6%. If spontaneous apoptosis was evaluated at lymphocyte subpopulation level, a broad variation was seen. Spontaneous apoptosis of CD4+ and CD8+ cells was similar to the unfractionated splenocytes, but it was much lower in the B cell population (1%), and much higher in the NK cells (6.5%), Treg cells (9.3%) and DC cells (11.3%). Low-dose irradiation decreased apoptotic frequency in most of the studied cell populations. Changes were the most pronounced for the NK and especially DC cells in the 0.01-0.1 Gy dose range, where a 25-50% drop in the proportion of apoptotic cells was seen when compared to unirradiated controls. The decreased apoptotic frequency of DCs after low dose irradiation persisted even 24 hours after irradiation. Irradiation with high doses (0.5 and 2 Gy) resulted in increased apoptotic frequencies in most of the studied cell populations. After irradiation with 2Gy, 23% of the total splenocytes were

apoptotic, and this represented a 6.6 fold increase in the apoptotic frequency. B cells were the most sensitive, 15.8 % of the total B cells were apoptotic after irradiation with 2 Gy, and this represented a 15.5 fold increase in the apoptotic frequency compared to the spontaneous apoptosis found in this subgroup of cells. The apoptotic frequency of the CD4+, Treg and CD8+ cells was intermediate, with 5.1, 4.1 and 6.9 fold increases, respectively. NK cells and DCs were the most resistant, where apoptosis frequency increased 3.26 and 2.25 fold compared to controls.

Alterations in the number of total splenocytes, as well as in the number and proportion of various splenocyte subpopulations were determined in the spleens of total-body irradiated mice 1, 3 and 7 days after irradiation. One day after irradiation an increase in the number of total splenocytes could be observed after irradiation with 0.01 Gy, although changes were statistically not significant. This increase was reflected in the number of most of the studied splenocyte subpopulations, as well. Irradiation with higher doses (0.05-0.5 Gy) induced a dose dependent decrease in total splenocyte number and changes were similar in NK, B and Treg cells. CD4+ and CD8+ cells were more resistant to low doses, while DCs were more sensitive. Irradiation with 2 Gy significantly decreased the relative number of all the studied cell populations, but major differences were seen among various lymphocyte subsets. The surviving fraction of total splenocytes was 25% of the control samples. CD8+ cell survival was similar, but that of CD4+, NK, DC and especially Treg was significantly higher. The ratio of CD4+ / CD8+ cells in the low dose range was similar to the control samples, but significantly increased after irradiation with 2 Gy, showing that CD8+ cells were more radiosensitive at higher doses than CD4+ cells. The ratio of CD4+ / CD4+CD25+ Treg cells in the low dose range showed mild, statistically insignificant alterations. However, after irradiation with 2 Gy this ratio decreased in a highly significant manner, showing the relative enrichment of Treg cells within the CD4+ population. B cells were significantly more sensitive to irradiation with 2 Gy than the other cell types (19.8 % surviving fraction).

Three days after irradiation, splenocyte numbers reached their minimum for most of the cell types. Total splenocyte numbers decreased by about 25% after irradiation with 0.05 Gy and did not further change significantly at 0.1 or 0.5 Gy. The relative numbers of Treg, NK and DC cells were lower after irradiation with 0.1 Gy than 0.5 Gy, suggesting potential low-dose hypersensitivity, although changes were statistically not significant. CD4+, CD8+ and B cell numbers showed a more uniform dose-dependent decrease after low-dose irradiation. The differential sensitivity of cells seen on day one after irradiation with 2 Gy persisted on day three, as well; the CD4+ / CD8+ and CD4+ / Treg ratios changed very little. The most resistant cells were the Treg, NK and DC cells with surviving fractions of 53%, 45% and 42% at 2 Gy. To better characterize the Treg cell population, the presence of Foxp3 marker was also evaluated on the CD4+CD25+ cells. The percent of CD4+CD25 bright and CD4+CD25+Foxp3+ cells within the total CD4+ population was very similar at all applied radiation doses. The presence of CD44+ memory T cells within the CD8+ population was also evaluated 3 days after irradiation. The proportion of memory T cells increased with doses but not in a linear manner (Table 4). Irradiation with 0.01-0.1 Gy induced a 2.5-3 fold increase and irradiation with 0.5-2 Gy resulted in a 4.5-fold increase in the ratio of memory CD8+ cells. Seven days after irradiation splenocyte numbers changed very little compared to day three. However, the quality of changes was different in the low and high-dose range. Cell numbers in the samples irradiated with higher doses (0.5 and 2 Gy) slightly recovered for most of the studied cell populations, statistically significant increases were detected in the CD4+ population ($p<0.01$ in the samples irradiated with 0.5 Gy at day 7 compared to day 3) and especially in the CD8+ population ($p<0.02$ in the samples irradiated with 0.5 and 2 Gy at day 7 compared to day 3). The fact that CD8+ cells after irradiation with 2 Gy recovered better than CD4+ cells was reflected in the CD4+ / CD8+ ratio as well, which tended to normalize by day 7. In the low-dose range recovery was absent for most of the cells, with the exception of DCs.

To investigate alterations in the cytokine expression profile of irradiated lymphocytes mice were irradiated with 0, 0.01, 0.05, 0.1 and 2 Gy gamma-radiations, and whole-cell RNA was isolated from the splenocytes. The relative amount of various cytokine transcripts was investigated by quantitative RT-PCR. In order to determine time-dependent alterations, we studied expression patterns at three different time points: 4, 24 and 72 hours after irradiation. The panel of investigated cytokines included Th1 or Th1-like (IL-2, IL-12, IFN-γ, LT-β or TNF-β, TNF-α), Th2 (IL-4, IL-6, IL-10), Th3 (TGF-β) cytokines, as well as cytokines with hematopoietic growth factor properties (GM-CSF, IL-5) and cytokine-like proteins with uncertain biological functions in lymphocytes (GDF15).

High-dose (2 Gy) irradiation induced strong alterations in the expression pattern of most cytokines with the majority of them being over-expressed. We could detect changes in the cytokine expression pattern after low-dose irradiation also, but changes were milder and less unequivocal than after high-dose irradiation. IFN-γ and to a lesser extent IL-2 expression decreased after low-dose irradiation and increased after 2 Gy during the whole investigation period, albeit changes were more pronounced four hours and 3 days after irradiation. IL-12 expression decreased after low and increased after high dose irradiation at an early time point (four hours) after irradiation and normalized by day 3. LTβ showed more than 50% reduction in its expression rate 3 days after high doses; low doses had no effect at any time point. IL-4 expression decreased four hours after both low and high-dose irradiation. Low-dose induced alterations persisted 3 days after irradiation, but IL-4 level went back to normal values after irradiation with 2 Gy. IL-6 expression showed a biphasic pattern: four hours after irradiation statistically significant increase was detected for all doses. Then, at day 1, IL-6 levels decreased at all radiation doses and tended to normalize at day 3. IL-10 was a late-reacting cytokine, since the most prominent dose-dependent increase was observed 3 days after irradiation. Low-dose irradiation significantly decreased GDF15 expression level on day 1, while irradiation with 2 Gy led to a persistent increase lasting up to 3 days. GM-CSF expression was less affected by low doses. Its expression increased in a biphasic way after irradiation with 2 Gy; it was most pronounced immediately after irradiation and at day 3. Both low and high doses heavily induced IL-5 expression at all of time points.

Next we focused our attention on radiation effects on regulatory T (Treg) cells which are considered as suppressive elements of the immune system. Mice were irradiated with 2 Gy. Radiation induced a massive total splenocyte loss after 1 day (4.24-fold decrease in cell number compared to non-irradiated animals), cell numbers continued to decrease until day 3 (5.38-fold decrease compared to control), and a moderate regeneration was noted by day 7 (3.78-fold decrease). Similar cell loss kinetics were observed within the CD4+ pool and the CD4+CD25+Foxp3+ Treg subpopulation, but the cell number losses were less pronounced (3.9-, 4.58-, 3.11-fold decrease in the CD4+ pool, as well as 2.92-, 3- and 1.98-fold decrease in the Treg pool). The data suggest that CD4+ lymphocytes are more radiation resistant than the total splenocyte pool, and within the CD4+ population, Treg cells are even more resistant. The different radiation sensitivity of the various lymphocyte subpopulations leads to the enrichment of Treg cells within the CD4+ population. In order to identify mechanisms responsible for Treg cell enrichment first we investigated the rate of Treg cell apoptosis in the CD4+ population following irradiation. Since radiation induced apoptotic cell death occurs relatively rapidly in the lymphocytes, apoptosis was studied 4 hours, 1 day and 3 days after irradiation. About 28% of total splenocytes were apoptotic four hours after irradiation showing an 8-fold increase above the background rate. Increased apoptotic rates although at much lower levels (3- and 2.9-fold increase) were still detected 1 and 3 days after irradiation. The onset of apoptosis within the CD4+Foxp3- population and CD4+Foxp3+ Treg populations was more moderate and slower: alterations reached their maximum value 24 hours after irradiation (5.4- and 3.4-fold increase compared to control values) and started to normalize by day 3 after irradiation. Spontaneous apoptosis of CD4+Foxp3+ Treg cells was higher than that of CD4+Foxp3- cells. However, it is important to note that the radiation induced increase in the rate of apoptosis was stronger in CD4+Foxp3- cells than in CD4+Foxp3+ Treg cells at all investigated time points. This suggests that Treg cells were less prone to radiation-induced apoptosis than effector T cells, with apoptosis being both less pronounced and less prolonged.

Irradiation of mice with 2 Gy resulted in a strong lymphopenia that might induce the homeostatic proliferation of the surviving immune cells to restore cell numbers. To investigate the potential role of homeostatic proliferation in the selective enrichment of Treg cells, we investigated differences in the proliferation rate of CD4+Foxp3- cells and CD4+Foxp3+ Treg cells by monitoring the expression of the intracellular proliferation marker Ki67. The fraction of proliferating CD4+ cells in the total CD4+ pool of the unirradiated mice was 3.4%, which, after a transient decrease at day 1 after irradiation, increased to 6.26% by day 3 and remained practically unchanged at later time points. The ratio of Foxp3- and Foxp3+ fractions within the proliferating CD4+ population was 74:26 (Foxp3- : Foxp3+) in the control animals, which gradually changed to 59:41 by day 11 after irradiation. The data demonstrate that in irradiated mice Treg cell proliferation had a more dynamic increase over time than that of Foxp3- CD4 cells, which, together with a lower susceptibility to radiation-induced apoptosis, resulted in a more efficient regeneration of the Treg pool. To test the

possibility that Treg cell enrichment might be the result of the conversion of CD4+Foxp3- T cells to CD4+Foxp3+ Treg cells, mice were intravenously transplanted with isolated, CFSE labeled, syngeneic effector T cells and 24 h later were irradiated with 2 Gy. Five days after irradiation, the phenotype of CFSE positive cells was characterized. The proportion of CFSE positive cells present in the spleen of both control (unirradiated) and irradiated animals was about 0.5% of the total splenocytes. The fraction of Foxp3+ Treg cells within the CFSE labeled effector T cells was 0.73% (±0.27) at the time of injection. In case of conversion, an increase in this fraction would have been expected. However, neither in the case of control, nor in the case of irradiated animals this fraction has significantly changed, indicating the lack of conversion of CD4+Foxp3- T cells to CD4+Foxp3+ Treg cells.

Radiation might modify Treg cell functions by altering the expression of cellular activation markers and by modifying cytokine expression. It was suggested that the presence of CTLA4 receptor protein on the cell surface was upregulated on activated Treg cells and the presence of GARP surface molecule was also associated with Treg activation. The fraction of Treg cells expressing CTLA4 was 5.3% in unirradiated control mice, which increased to 10.5% upon irradiation. Interestingly, CTLA4 expression also increased on CD4+Foxp3- T cells. Concerning GARP, 65% of CD4+Foxp3+ Treg cells from control, unirradiated animals were GARP positive, which increased to 80% after irradiation. These *in vivo* data suggest the moderate upregulation of Treg cell activation markers following irradiation.

Next we tested how irradiation influenced IL-10 and TGF-β mRNA levels in Treg cells and CD4+Foxp3- T cells. These two cytokines were chosen because they are considered to be particularly important in mediating the suppressor activity of Treg cells. Basal IL-10 mRNA expression was higher in Treg cells than in CD4+Foxp3- T cells. Irradiation induced a moderate (~1.2-fold) upregulation of IL-10 mRNA expression in Treg cells. Interestingly, upregulation of IL-10 was slightly stronger (~1.4-fold) in CD4+Foxp3- T cells. The basal levels of TGF-β expression was moderately higher in CD4+Foxp3- T cells than in Tregs and radiation further upregulated it while it did not influence TGF-β expression in Treg cells. These data suggest that irradiation resulted in a more pronounced upregulation of IL-10 and TGF-β expression in CD4+Foxp3- T cells than in Treg cells. It should be noted however, that the detected cytokine alterations at mRNA level were relatively moderate and they do not necessarily represent actual changes in the level of secreted cytokines. Finally, we have investigated whether radiation modified the suppressive function of Treg cells on CD4+CD25- effector T cells. Treg cells were isolated from the spleens of irradiated and control mice three days after irradiation and were incubated under *in vitro* conditions with effector T cells from unirradiated mice. Effector T cell proliferation was investigated as functional endpoint. Treg cells isolated from unirradiated control mice reduced effector T cell proliferation to 6%, while Treg cells derived from irradiated animals to 14% ($p=0.001$). These data demonstrate that the proliferation rate of effector T cells cocultured with irradiated Treg cells was more than two-fold higher than the proliferation rate of effector T cells cocultured with non-irradiated Tregs. This is a significant reduction in the suppressive capacity of irradiated Treg cells.

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