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Research Article

Transcriptional response to ionizing radiation in lymphocyte subsets

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Abstract. Human lymphocyte subpopulations differ in their cellular responses to ionizing radiation. To shed light on the molecular basis of this effect, we characterized the transcriptional response to 1 Gy X-rays of CD4+ T lymphocytes. Of 18,433 genes tested, 102 were modulated more than 1.5-fold. The majority of the strongly activated genes were p53 targets involved in DNA repair and apoptosis. The expression of three of these genes was further tested by quantitative RT-PCR in lymphocyte subpopulations [CD4+ and CD8+ T, CD19+ B, CD56+ natural killer cells and peripheral blood lymphocytes (PBLs)] from ten adult donors. In contrast to *DDB2*, *TNFRSF10B* and *BAX* were differentially modulated among the subpopulations and the PBLs, being more activated in irradiated CD19+ B and CD8+ T lymphocytes. The level of *BAX* activation in the various subpopulations correlated with the sensitivity of the cells to radiation, suggesting its possible role in the differential radiosensitivity of hematopoietic cell subsets.

Key words. Radiation; lymphocyte; cDNA microarray; quantitative real-time RT-PCR; *BAX*, *TNFRSF10B*; B, T, NK lymphocytes.

In eukaryotic cells, ionizing radiation triggers various signaling pathways that converge to the induction of repair mechanisms and regulation of cell cycle checkpoints. Failure to achieve correct repair might lead to cell death, premature senescence, or carcinogenesis [1]. The dose required to induce cell death in a defined percentage of cells in a given cell population defines the level of radiosensitivity of that cell population [2]. Hematopoietic cells are particularly sensitive to radiation. Indeed, individuals exposed to whole-body irradiation develop radiation syndromes affecting different organs: death from the hematopoietic syndrome occurs at doses lower than those causing death from the gastrointestinal, cutaneous, or neurological syndromes. Differences in the hematopoietic compartments are also observed. Indeed, a decline in the count of circulating lymphocytes is seen during the early phase (within 3 days) in individuals exposed to 1–2 Gy of acute external radiation [3]. The neutrophils, thrombocytes and reticulocytes disappear progressively later [3]. This compromised hematological status might increase susceptibility of individuals to opportunistic infections [3]. The risk of infection due to deficiency in neutrophil and lymphocyte activity is also increased in patients undergoing radiation therapy [4]. In addition, several disorders of hematopoietic origin such as anemia, autoimmune disease, and leukemia after external exposure to radiation have been reported in epidemiological studies [5, 6].

The first large screening by gene expression profiling to unravel part of the molecular mechanisms of the radiation response in hematopoietic cells has been performed on circulating peripheral blood lymphocytes (PBLs) that had been isolated from healthy donors and subsequently irra-

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diated *ex-vivo*. These studies have shown a linear dose-response, with activation of *DDB2*, *CDKN1A* (*CIP1/WAF1*), and *XPC* seen at doses of γ rays as low as 0.2 Gy [7]. These data have been confirmed and further extended by other groups [8–10]. PBLs constitute a heterogeneous mixture of subpopulations. Their major constituting groups are the CD3+ T lymphocytes (further separated into CD4+ and CD8+), the CD19+ B lymphocytes and the CD56+ natural killer (NK) cells. Sensitivity to ionizing radiation varies among these subpopulations [11–13]. These observations, mainly obtained *in vitro*, originate from colony formation assays or micronucleus measurements. Among PBL subpopulations, NK cells are highly resistant to the effects of irradiation compared to the T and B lymphocytes [reviewed in ref. 13]. One factor accounting for intrinsic radiosensitivity has been related to the basal expression level of specific genes. For example, the content of the anti-apoptotic BCL2 protein in neutrophils, lymphocytes, and monocytes inversely correlates with Fas-mediated cell death susceptibility in these three cell types [14]. Also, the higher radiosensitivity of B lymphocytes has been linked to a lower activity of the DNA repair protein DNA-PK. These lymphocytes express an inhibitory variant form of the protein Ku86 with low ability to recruit DNA-PK, which competes with both Ku86 and Ku70 in the binding of DNA-PK [15].

As the molecular basis of the differential radiation sensitivity among lymphocyte subpopulations remains unclear, we investigated how the profile of gene expression (18,433 unique sequences on microarrays) was affected by ionizing radiation in a CD4+ T cell lymphocyte subpopulation. We next studied the expression of three of the most strongly activated genes, *DDB2*, *BAX*, and *TNFRSF10B*, in the PBL population from ten donors, and the level of regulation was compared to that seen in the CD4+, CD8+, CD19+ and CD56+ subpopulations.

Materials and methods

Cell purification, culture, and irradiation

Peripheral blood mononuclear cells (PBMCs) were isolated from the venous blood of ten healthy blood donors (five women and five men, aged 43 ± 13 years) by density centrifugation over Ficoll Paque (Sigma, St. Louis, Mo.; density 1.077 g/ml). PBLs were recovered from PBMCs following adherence of monocytes on the surface of plastic flasks at 37 °C for 1 h. Lymphocyte subpopulations (CD4+ and CD8+ T, CD19+ B, and CD56+ NK cells) were purified from PBLs using super-paramagnetic anti-CDs MACS MicroBeads antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany), as suggested by the manufacturer.

After purification, cells were cultured in RPMI 1640 medium (Invitrogen, Merelbeke, Belgium) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (Invitrogen) at 37 °C in a 5% CO_2 and 95% humidity incubator. Irradiation was performed the next day, 24 h after blood collection at 22 °C in 75-cm^2 flasks. Irradiation was done using the Pantak RX, 250 kV – 15 mA – 1 mm Cu filter installation with a dose rate of 0.3 Gy/min, determined by using a 0.6-cm^3 ionization chamber (NE 2571), connected to a dosimeter (Farmer dosimeter 2570).

Flow cytometry analysis

For population purification control, aliquots from unseparated and purified populations were centrifuged at 1500 rpm for 5 min and labeled with the fluorescein-labeled anti-human CDs specific antibody (IgG1 mouse; Analis, Namur, Belgium) diluted according to the manufacturer's suggestions in phosphate-buffered saline (PBS) with 0.5% FBS. Cells were incubated at room temperature for 20 min and centrifuged again before proceeding to flow cytometry analysis. Flow cytometry analyses were performed on an Epics XL fluorescence-activated cell sorter (Coulter Beckman). Purities of cell subpopulations were 97%, 86%, 82%, and 92% for CD4+, CD8+, CD19+, and CD56+ cells, respectively.

RNA isolation

Cells were centrifuged at 3000 rpm for 10 min. The pellet was washed in 1 ml cold PBS and resuspended in 1 ml Trizol (Invitrogen). The suspension was then transferred to an RNase-free Eppendorf tube, passed through a 20-gauge needle and stored at –70 °C until RNA extraction was performed according to the manufacturer's recommendations. RNA quality was assessed with an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, Calif.).

Microarray analysis

Five micrograms of total RNA were reverse transcribed with an oligo-dT primer linked to a T7 promoter sequence and linearly amplified using an *in vitro* transcription reaction as previously described [16]. The cDNA was labeled with Cy3 (sham irradiated control) or Cy5 (irradiated) and then hybridized to four separate glass slides each containing an average of 4,300 unique cDNAs spotted in duplicate. The lists of the genes included on these slides are deposited in Array Express (accession numbers A-MEXP-104, A-MEXP-105, A-MEXP-106, A-MEXP-107). Microarray experiments followed the procedures previously described [16]. Fluorescence intensities were normalized by applying the Lowess algorithm [17]. Slice analysis was applied as criteria for selection of modulated genes. Data point population of 50 and data keep range over 2 standard deviations were used as filter [17]. Functional analysis groups were defined using Database for Annotation, Visualization and Integrated Discovery (DAVID: http://apps1.niaid.nih.gov/david/). Minimum Information About a Microarray Experiment (MIAME)-

compliant intensity has been deposited in Array Express (accession number E-MEXP-320).

Quantitative real-time RT-PCR analysis

RNA samples were purified on silica-membrane-based columns (Promega, Leiden, The Netherlands) in accordance with the instructions of the supplier. RNA quantity was assessed with Nanodrop (Isogen Life Science B. V., Maarssen, The Netherlands) and quality verified by gel electrophoresis. Total RNA (0.5 μg) was reverse transcribed into cDNA by Multiscribe Reverse Transcriptase, using the Reverse Transcription kit (Applied Biosystems, Foster City, Calif.) with cycling conditions of 10 min at 25 °C, 45 min at 48 °C and 5 min at 95 °C. One-hundredth of the resulting synthesized single-strand cDNA was used for each PCR reaction in the presence of 3 μM specific primers and Syber green PCR master mix (Sigma). Quantitative PCR was performed on a GeneAmp 5700 Sequence Detection System (Applied Biosystems), with cycling conditions of 2 min at 50 °C, 10 min at 95 °C and 40 times 15 s at 95 °C, 1 min at 60 °C. After the last cycle, the temperature was progressively raised to provide dissociation curves that allow assessment of the purity of the amplified product. The average PCR amplification efficiency for all the primers used was above 90%. Each PCR was performed in duplicate or triplicate and analyzed with fixed baseline threshold that served to define the threshold cycle (Ct) of each condition. Quantities were calculated

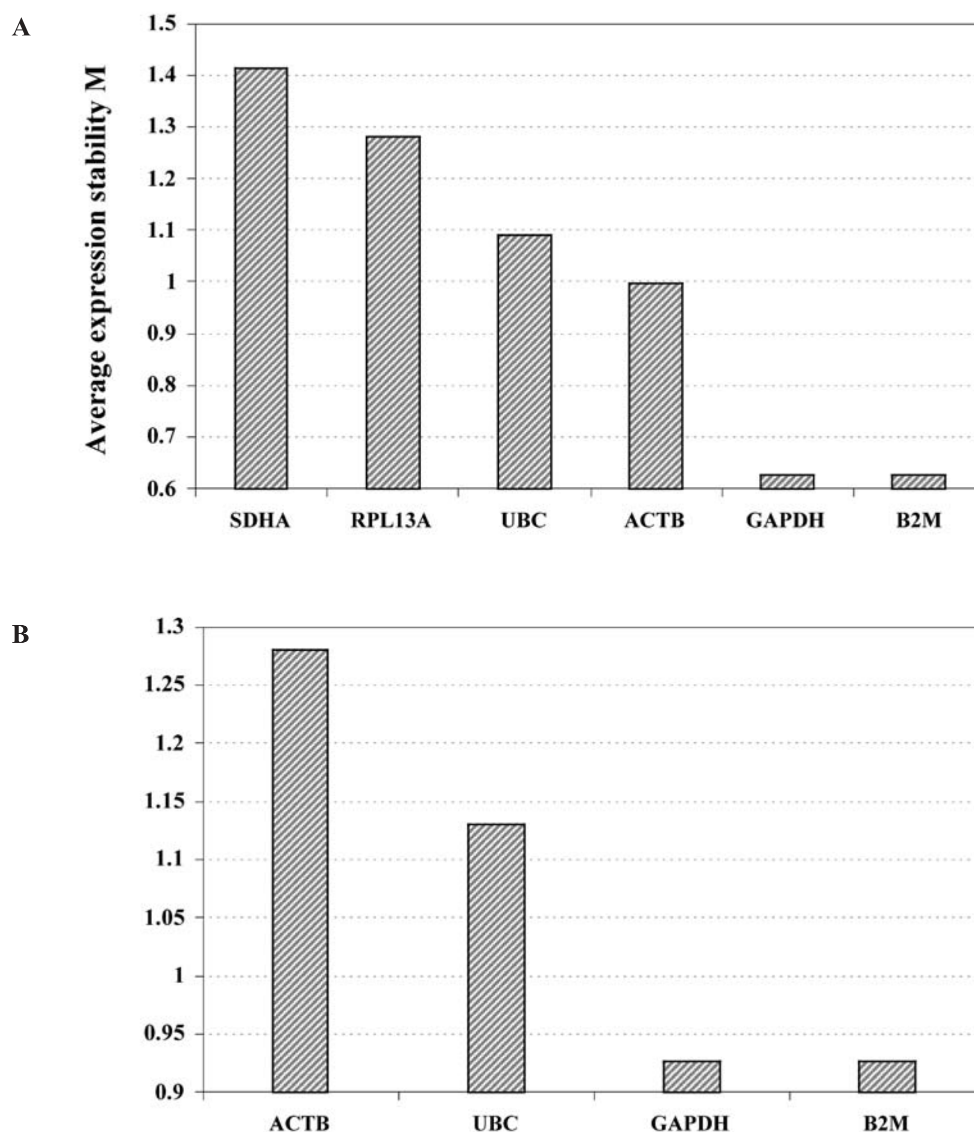

Figure 1. Average expression stability values (M) of selected housekeeping genes. (*A*) Initial analysis included six housekeeping genes measured in PBLs, CD4+, CD8+, CD19+, and CD56+, cells isolated from one donor, in the two conditions (irradiated with 1 Gy and sham) after 8 h (10 samples). (*B*) A second analysis was done using the four most stable genes (ACTB, UBC, GAPDH, and B2M) in a larger number of samples (50 samples) prepared from five donors.

Table 1. List of primers and housekeeping genes used in real-time quantitative RT-PCR for analysis of radiation-induced modulation in PBLs and in lymphocyte subsets.

Short name	Forward primer	Reverse primer	Identifier
B2M	5′TGCTGTCTCCATGTTTGATGTATCT3′	5′TCTCTGCTCCCCACCTCTAAGT3′	NM_004048
GAPDH	5′CAACTACATGGTTTACATGTTC3′	5′GCCAGTGGACTCCACGAC3′	NM_002046
UBC	5′ATTTGGGTCGCGGTTCTTG3′	5′TGCCTTGACATTCTCGATGGT3′	M26880
ACTB	5′TACCCACACTGTGCCCATCTAC3′	5′GAACCGCTCATTGCCAATG3′	NM_001101
RPL13A	5′CCTGGAGGAGAAGAGGAAAGAGA3′	5′TTGAGGACCTCTGTGTATTTGTCAA3′	NM_012423
SDHA	5′TGGGAACAAGAGGGCATCTG3′	5′CCACCACTGCATCAAATTCATG3′	NM_004168
DDB2	5′ATTGAGGACGATCGACGTGTTC3′	5′TGGCCCACACCTTCTTTAGTG3′	NM_000107
DR5	5′CTCTTTCCAGCAAACCCTTCTC3′	5′ACCAGTCCCGGAACAAAACAC3′	H68181
BAX	5′CCAGTTGAAGTTGCCGTCAGA3′	5′TGGAGCTGCAGAGGATGATTG3′	AI025937

from the mean of the Ct_s replicates applying the delta Ct method, which uses the formula $q = x^{(\Delta Ct)}$, where x represents the PCR efficiency (x = 2 for 100% efficiency, x = 1.90 for 90% efficiency) of each gene, ΔCt the difference between the tested Ct and the lower Ct among the overall conditions, and q the relative expression level. Thereafter, values were normalized considering the *UBC, GAPDH*, and *B2M* internal control gene levels (see below) [18]. Briefly, we first selected stable internal control genes by comparing their M values. The M value is the arithmetic mean of the pairwise variation (standard deviation of a particular gene) over the samples [18]. The selection proceeded in two steps. First, the six following housekeeping genes, *SDHA*, *RPL13A*, *UBC*, *ACTB*, *GAPDH*, and *B2M*, were analyzed by quantitative real-time RT-PCR in all lymphocyte subpopulations (PBLs, CD4+, CD8+, CD19+ and CD56+) from one donor irradiated with 1 Gy or sham, harvested after 8 h (fig. 1a). A list of these primers can be found in table 1. Four of these genes, *UBC*, *ACTB*, *GAPDH*, and *B2M,* with the lowest M values (which express the highest stability) (fig. 1a) were further measured by quantitative real-time RT-PCR in all lymphocyte subpopulations from five of the ten donors (fig. 1b). The three best-performing housekeeping genes, *UBC*, *GAPDH*, and *B2M*, were further measured in all ten donors and their expression levels applied for normalization among donors and conditions. A normalization factor for each sample was calculated using the geometric mean of the expression level of *UBC, GAPDH*, and *B2M*. Expression ratios were defined as the ratio of cDNA concentrations in samples from irradiated and sham-irradiated cells.

Statistical analysis

Statistical analysis was performed with GraphPad InStat software version 3.00 for Windows 95 (San Diego, Calif. www.graphpad.com). Differences between pairwise samples were measured with a paired t-test. ANOVA and Tukey post-test were applied for comparing more samples. The significance level was set at 0.05.

Results

Changes in the transcriptional profile of CD4+ T lymphocytes induced by ionizing radiation

The profile of gene expression had previously been investigated in primary CD4+ T lymphocytes purified from one healthy donor 8 h after *ex vivo* irradiation with 1 Gy X-rays, using microarrays containing 13,825 cDNA sequences [16]. The selected dose and time corresponded to the LD_{50} and to the peak of activation of a marker gene (*DDB2*), respectively, as deduced from our previous analysis [16]. In the present study, we extended the number of genes under investigation by adding an additional set of 4,608 unique cDNA sequences. A total of 18,433 unique sequences were screened for their transcriptional response to ionizing radiation. Compared to our previous analysis [16], an alternative normalization method (Lowess) was used, leading to more stringent criteria for the inclusion of genes regulated by radiation. Signals with an intensity of two standard deviations above the background and with high reproducibility (coefficient of variance <0.5) among duplicates were retained. Modulated genes were selected by slice analysis (see Materials and methods), excluding genes corresponding to hypothetical proteins (39 genes). The total number of regulated genes amounted to 102, with bias toward up-regulated genes (75 compared to 27 that were down-regulated) (tables 2, 3; for supplementary material see: http://www.sckcen.be/sckcen_en/activities/research/radiationprotection/radiobio/projects.shtml#mori). In an attempt to distinguish functional pathways triggered by radiation, activated and repressed genes were grouped into three levels of modulation and classified for biological function using Gene Ontology (GO) annotations. Their dependence on p53 activation, as documented in the literature [19–26], was also indicated. The activated genes were distributed in the three levels of modulation, representing 21.3%, 17.3% and 61.4% of the high (>3-fold), intermediate (between 2.5- and 3-fold), and low (between 1.5- and 2.5-fold) levels, respectively. In contrast, the totality of the repressed genes was in the low level of modulation (fig. 2).

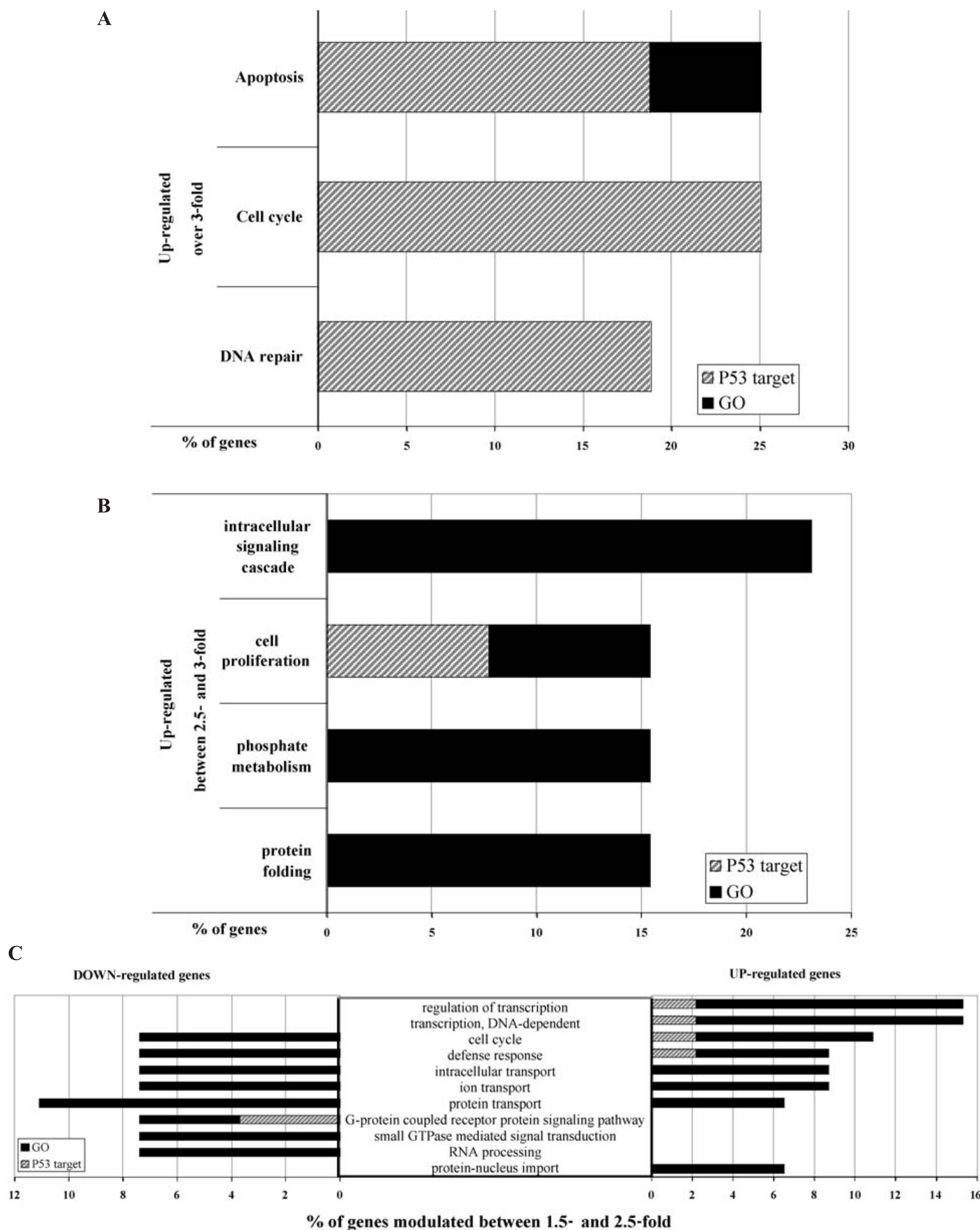

Figure 2. GO classes and p53 targets in the up- and down-regulated genes. Up- and down-regulated genes were divided according to their levels of modulation and the respective GO classes were derived from analysis with the DAVID-web tool. p53 targets were defined from the literature. GO and p53 target bars were overlapped to show the relative percentage of p53 targets in each GO functional class. A complete stripped bar indicates that 100% of the genes are p53 targets. A complete black bar indicates that no p53 targets are within the GO class. Results are expressed as percentage of the total number of genes in each level of modulation. (*A*) Histograms for genes activated more than 3-fold (total 16 genes). (*B*) Histograms for genes activated between 2.5- and 3-fold (total 13 genes). (*C*) Histograms for genes up and down modulated between 1.5- and 2.5-fold (46 and 27 genes, respectively).

Table 2. Genes up-regulated by irradiation in primary CD4+ T lymphocytes.

Genebank accession number	Short name	Array (this study)	p53 target	Array PBL (others)	RT-qPCR (this study)
N98621	GADD45A	7.45	yes[19–21]	2.25 [8]	13.9 [16]
AI935984		1.74		4.06 [10]	
AI683074	FDXR	6.22	yes[23]	1.92 [8]	
				2.34 [10]	
R01076	DDB2	5.71	yes[20, 21]	3.33 [7]	8.5 [16]
NM_000107		5.21		2.14 [8]	
				2.39 [10]	
AA046808	RPS27L	4.65			4.6
AA927505		2.95			
T82974	PCNA	4.63	yes[20]	2.44 [7]	
				2.20 [8]	
H53043	PHP14	4.60			3.1
AA706226	NRG2	4.39			
W46835	FHL2	4.13	yes[20]	9.30 [9]	
				3.06 [10]	
AA609473	KIF9	4.08			
H68181	TNFRSF10B	4.02	yes[19]	3.88 [7]	11.7 [16]
				1.43 [8]	
				2.30 [10]	
N68917	SESN1	3.60	yes		12.4 [16]
AI572037		2.94			
R87514	CDKN1A	3.54	yes[19-21]	4.05 [7]	8.9 [16]
L26165		2.28		1.95 [8]	
W39472		1.92		4.23 [10]	
BG164410	TRIM22	3.18	yes[22]	3.25 [7]	
				1.55 [10]	
AW409774	ASTN2	3.08			
H60986	RNAH	3.07		1.88 [10]	
AA492031	C9	3.06			2.1
R14996	CCNG1	2.89	yes[21]	2.61 [7]	
BC000196		2.35		1.43 [8]	
				2.0 [9]	
				1.71 [10]	
AA007354	PHLDA3	2.88			
U53470	INPP5D	2.86		–1.09 [10]	
NM_002738	PRKCB1	2.78			
BG326971	FKBP8	2.71			
N52496	BTG3	2.70		1.70 [8]	
				1.11 [10]	
N50998	SYN2	2.70			
N99925	TPM1	2.69			
U33821	TAX1BP1	2.65		1.38 [8]	
				1.13 [10]	
H22512	BRUNOL5	2.61			
W04507	PFDN4	2.56			
NM_003184	TAF2	2.52			
N52259	STX6	2.52		1.54 [10]	
N54067	MAP4K4	2.49			
BE350101		1.69			
H58959		1.69			
BG619272	HLA-DQB1	2.47		–1.32 [7]	
				–1.19 [10]	
AA398283	ELL	2.44		2.41 [7]	
AW593261	GTPBP2	2.40			
T84808	IER5	2.38			
AI928745	POU3F4	2.38			
AL533554	TSN	2.36			
AI671762	RAB3IP	2.35			
AI878864	ATSV	2.31			
AI025937	BAX	2.30	yes	1.63 [8]	10.7 [16]
				1.92 [10]	
BG034382	OS4	2.29	yes[20]		

Table 2. Genes up-regulated by irradiation in primary CD4+ T lymphocytes.

Genebank accession number	Short name	Array (this study)	p53 target	Array PBL (others)	RT-qPCR (this study)
N92797	PFDN2	2.24			
H64024	PRKAB1	2.22	yes[20]	2.35 [10]	
N65950	ARHGEF3	2.19			
NM_014635	GCC2	2.18			
NM_004628	XPC	2.17	yes[24]	3.06 [10]	
AA448972	MUSK	2.13			
AA603466	PTEN	2.06	yes[21]	1.44 [7]	
AI912413		1.68		1.72 [8]	
W96187	SCN8A	2.06			
NM_005125	CCS	2.01			
BG764734	GPX1	1.98	yes[25]	–1.33 [7]	
N58247		1.93		2.40 [9] 1.31 [10]	
AA013091	RGL	1.95			
U72069	KPNB2	1.95		1.10 [8] –1.26 [10]	
N34687	NIN	1.95			
R17138	F8	1.93			
N69679	ATF3	1.90	yes[20]	1.12 [7] 1.32 [8] 2.93 [10]	10.1 [16]
R68095	PTP4A1	1.89		2.17 [10]	
R12359		1.86			
NM_032626	RBBP6	1.88		–1.15 [10]	
H06292	SATB1	1.87		1.03 [7]	
BC010090	ACTR1B	1.86			
NM_004584	RAD9A	1.85			
R56692	SFRS2IP	1.83			
N20922	GOLGA4	1.78		2.08 [8]	
H95236		1.65		1.13 [10]	
BE869155	MLF2	1.76		1.97 [8] 1.34 [10]	
AA406323	TNFRSF6	1.75	yes[19-21]	1.47 [8] 2.61 [10]	
AI765790	TPR	1.74			
R60661	ATP10D	1.74		–1.10 [10]	
R82178		1.59			
R54792	PCNP	1.72		1.10 [10]	
R59506	CEB1	1.72			
H09985	RPS6KA5	1.70			
N89774	TCF20	1.68			
AI867198	MRPS6	1.67			
AA807633	BTG1	1.66		1.63 [8] 1.34 [10]	
AA010370	NXF3	1.62			
AI042300	NAP1	1.55			
AA480086	SEI1	1.54			

The table indicates Genbank accession numbers, short names, values measured with our in-house arrays [Array (this study)], the known p53 targets, the expression levels measured in PBLs by other groups [Array PBL (others)], and expression levels measured by us in RT-quantitative PCR [RT-qPCR (this study)]. p53 targets were derived from the literature or DAVID annotation tool. The three columns, 'p53 target', 'Array PBL (others)', and 'RT-qPCR (this study)' show the corresponding source reference in brackets. Some of the genes are represented more than once by cDNAs corresponding to different Genbank accession numbers.

Among the most strongly activated genes (over 3-fold modulation), three functional classes, namely apoptosis, cell cycle, and DNA repair, were represented. In this range of activation, the p53 pathway was clearly predominant, since 56% of the genes (9 out of 16) represented known p53 targets (fig. 2a). However, other pathways may significantly contribute to the transcriptional response to radiation, as demonstrated by the fact that, in the lower levels of modulation (below 3-fold), 80% of the activated and 97% of the repressed genes were not known p53 targets (tables 2, 3).

Genes activated at an intermediate level (between 2.5- and 3-fold) were grouped into functional classes corresponding mainly to the intracellular signaling cascades,

Table 3. Genes down-regulated by irradiation in primary CD4+ T lymphocytes.

GenBank accession number	Short name	Array (this study)	P53 target	Array (PBL others)	RT-qPCR (this study)
AA150500	ISG20	–2.21		–1.13 [10]	–3.3
R87311	AFAP	–2.14			
BG256267	MYC	–2.13		–1.49 [8]	–2.5
AA235530		–2.10		–2.27 [10]	
AA448983	RRP46	–2.00			
R56393	ARHGEF1	–1.83			–1.8
N20321	TOMM40	–1.80			
N47076	PAICS	–1.79		–1.41 [8] –1.10 [10]	
H58452	SNRP70	–1.78			
T97947	CNN2	–1.78			
AA707871	FCHO1	–1.78			
H50933	ATP2A3	–1.77		–1.27 [10]	
R13806	PES1	–1.75			
N39592	PSAT1	–1.74			
BG400371		–1.67			
N71460	RGS14	–1.73	yes[26]		
BC001454	PCK2	–1.70			
AA418027	RAB4B	–1.69		–1.30 [10]	
W48626	HLA-A	–1.69		1.09 [10]	
H29049	COPE	–1.68		1.08 [10]	
NM_002539	ODC1	–1.68		–1.03 [7]	
N78824		–1.56		–1.50 [8] –1.07 [10]	
AI824470	PARVG	–1.67			
NM_014767	SPOCK2	–1.63			
AA460688	NPFF	–1.59			
N74265	PLOD3	–1.57			
H51215	GDI1	–1.57			
R18466	RAVER1	–1.56			
H73256	LSP1	–1.55		1.13 [10]	
N62165	OSGEP	–1.52			

The table indicates Genbank accession numbers, short names, values measured with our in-house arrays [Array this (study)], the known p53 targets, the expression levels measured in PBLs by other groups [Array (PBL others)], and expression levels measured by us in RT-quantitative PCR [RT-qPCR (this study)]. p53 targets were derived from the literature or DAVID annotation tool. The three columns, 'p53 target', 'Array (PBL others)', and 'RT-qPCR (this study)' show the corresponding source reference in brackets. Some of the genes are represented more than once by cDNAs corresponding to different Genbank accession numbers.

but also cell proliferation (100% p53 targets), phosphate metabolism, and protein folding (fig. 2b). The lowest level of modulation regrouped both activated and repressed genes of which half were classified in similar functional classes related to the cell cycle, the defense response, as well as intracellular, ion, and protein transport (fig. 2c). However, three functional classes (protein-nucleus import, regulation of transcription, DNA-dependent transcription) were exclusively represented in the activated genes, while three classes corresponding to G protein signaling transduction (G-protein-coupled receptor protein signaling pathways and small-GTPase-mediated signal transduction) and RNA processing exclusively comprised down-regulated genes.

We have previously validated by real-time RT-PCR the levels of altered expression of several genes, including *GADD45A*, *DDB2*, *TNFRSF10B, SESN1*, *CDKN1A*, *BAX* and *ATF3* [16]. In this study, we tested by real-time RT-PCR the expression of six additional genes, among which *PHP14*, *C9*, and *RPS27L* were activated 3.1-, 2.1- and 4.6-fold, respectively, while *MYC, ISG20,* and *ARHGEF1* were repressed 2.5-, 3.3-, and 1.8-fold, respectively (tables 2, 3). Together, these results confirmed the good correlation between values from microarrays and those from real-time RT-PCR, although microarray data tended to be slightly underestimated. In addition, radiation-induced repression of *ISG20*, verified by real-time RT-PCR in primary CD4+ T lymphocytes from four additional donors submitted to the same irradiation treatment, gave values comparable to the above-mentioned results (–3.95 ± 1.15 SE, data not shown).

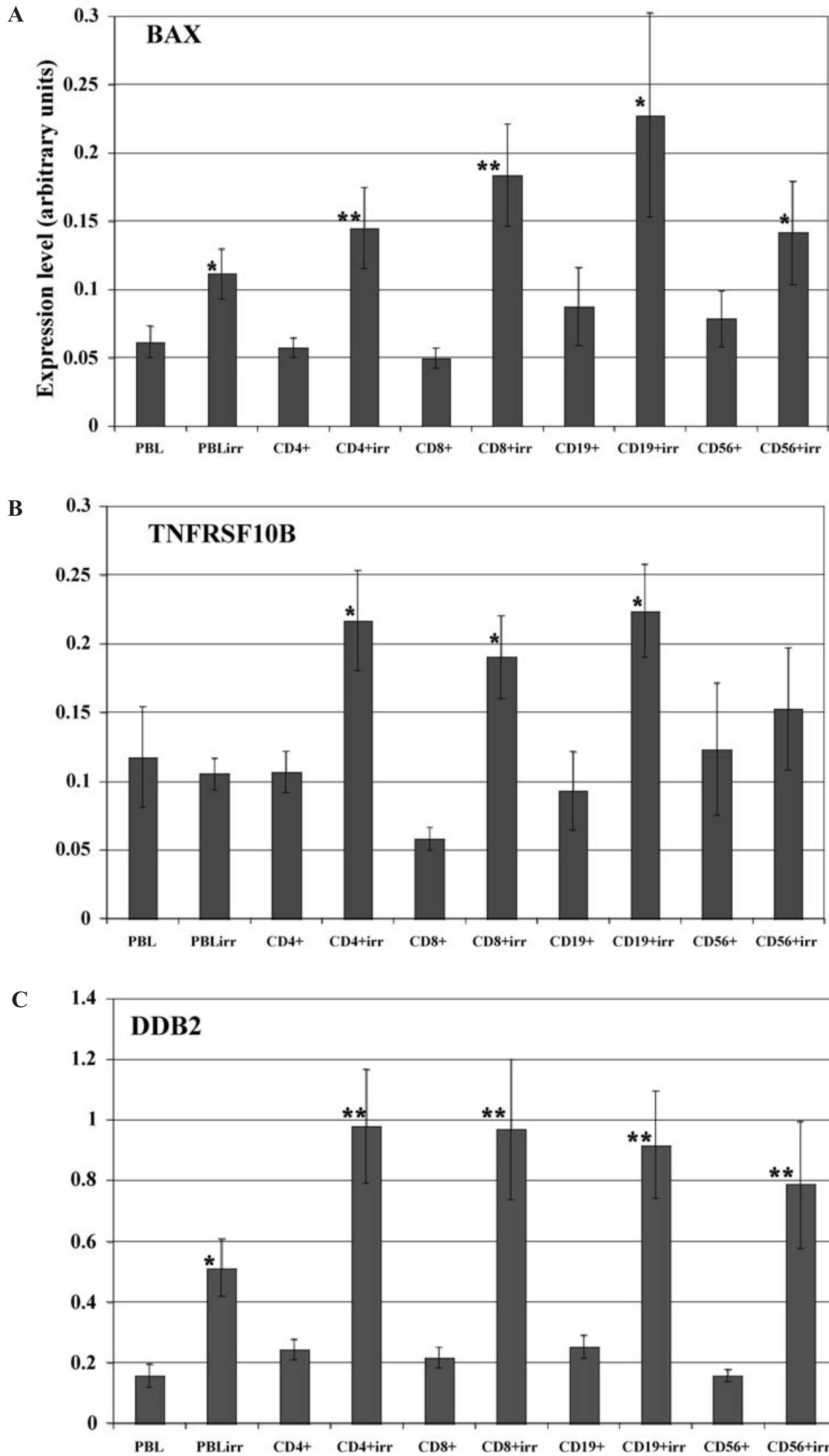

Figure 3. Gene expression levels measured by RT-qPCR of three p53 targets, *BAX* (*A*), *TNFRSF10B* (*B*), and *DDB2* (*C*), in PBL, CD4+, CD8+, CD19+, and CD56+ subpopulations, either in sham or irradiated (irr) conditions (1 Gy after 8h). Values represent the mean ±SE of ten different donors. * $p < 0.05$, ** $p < 0.01$ for irradiated versus sham

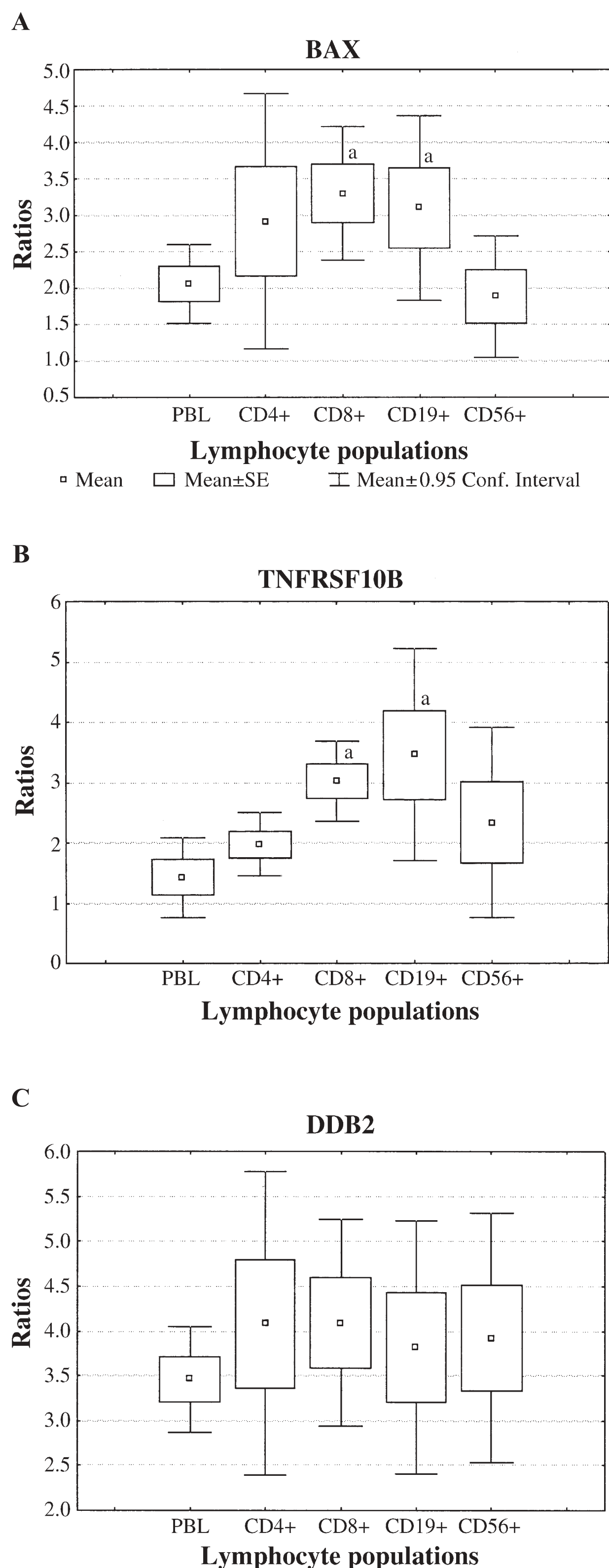

Figure 4. Relative expression (fold modulation ratio) level of three p53 targets, *BAX* (*A*), *TNFRSF10B* (*B*), and *DDB2* (*C*), in PBL, CD4+, CD8+, CD19+, and CD56+ subpopulations. Ratios were calculated in all samples from irradiated and sham treatment for each donor. Values represent the mean ± SE of ten different donors. a, $p < 0.05$.

Ionizing radiation differentially affects the expression of p53-dependent apoptotic genes in lymphocyte subsets

As the major pathway activated by radiation appears to be regulated by p53 in CD4+ T lymphocytes, we next investigated whether it was activated at similar levels in the other lymphocyte subpopulations. To address this point, three genes were selected among the two most represented functional classes activated in the CD4+ T lymphocytes (DNA repair and apoptosis). The expression levels of the two proapoptotic genes, *BAX* and *TNFRSF10B* and the DNA repair gene *DDB2* , were measured in PBLs, CD4+, CD8+, CD19+, and CD56+ lymphocytes 8 h after 1 Gy X-ray irradiation. The variability among individuals was assessed by measuring the response from ten healthy donors.

One-way ANOVA with Tukey post-test showed no significant differences in the basal levels of expression for *BAX* ($p = 0.515$), *TNFRSF10B* ($p = 0.568$) or *DDB2* ($p = 0.162$) (fig. 3). In contrast, radiation increased the expression of the three genes with significantly different amplitudes in the lymphocyte subtypes. Globally, for the three genes, the levels of expression attained after radiation were higher in the isolated subpopulations than in PBLs, and slightly lower for the CD56+ NK cells than for the other lymphocyte populations. Among the latter, there was no clear difference for *TNFRSF10B* and *DDB2*. In contrast, *BAX*-induced expression was higher in CD19+ B and CD8+ T lymphocytes compared to CD4+ T cells.

PBLs represent an easily accessible source of cells to test for biomarkers of exposure. However, as the expression of the p53 target genes in this study seemed to be higher in the B and T lymphocyte subpopulations compared to PBLs, we next assessed whether determination of their level of activation in separate subpopulations would bring any substantial advantage over the total PBLs, also taking into account the individual variability. The relative expression (fold modulation ratio) was calculated in all samples from irradiated and sham treatment for each donor and the mean of increased modulation of PBLs was compared to each of the lymphocyte subpopulations. As shown in figure 4a and 4b, a significant ($p < 0.05$) amplitude of modulation of the apoptotic genes *BAX* and *TNFRSF10B* was found in pairwise comparison of CD19+ and CD8+ [mean = 3.11 and 3.30, respectively, compared to PBLs (mean = 2.06) for *BAX*, and mean = 3.46 and 3.02, respectively, compared to PBLs (mean = 1.43) for *TNFRSF10B*]. In contrast, no significant changes were seen in the expression of *DDB2* between PBL and any of the subpopulations considered (fig. 4c). These results indicated that analyzing the regulation of p53-dependent proapoptotic genes in CD19+ B and CD8+ T lymphocyte subpopulations rather than in PBLs might increase their sensitivity as markers for radiation exposure.

Discussion

Changes in gene expression profiles associated with exposure to ionizing radiation vary according to several parameters, including genetic background, tissue, cell type, dose/dose rate, and quality of radiation [reviewed in ref. 27]. In this study, the alteration of the transcriptional program of primary CD4+ T lymphocytes exposed to 1 Gy X-rays was studied. Among the 18,433 unique sequences screened on the microarrays, 141 genes (of which 102 corresponded to known proteins) only were identified as responsive to radiation, with bias towards up-regulation. Genes that were the most highly activated (above 3-fold) were distributed into three functional classes, annotated in GO as apoptosis, DNA damage recognition/repair, and cell cycle. Several genes were represented in more than one class, particularly in the case of the cell cycle functional class including genes such as *GADD45A* and *PCNA* on the one hand, and *CDKN1A* on the other hand, which also play important roles in DNA repair and apoptosis, respectively. The irradiation protocol did not induce exit from the G0/G1 phase of the cell cycle that characterizes freshly isolated lymphocytes cultured in the absence of stimulating conditions, as verified by flow cytometry (data not shown). It is thus unlikely that cell cycle genes activated by irradiation are actually playing an active role in regulating the cell cycle of CD4+ T lymphocytes. We can deduce therefore, that only two functional classes were represented in the most strongly activated genes, corresponding to apoptosis and DNA repair and in which the p53 pathway plays a preponderant role. Among the latter class, *GADD45A*, which was the most highly modulated gene, might mediate during the radiation response cross-talk between DNA repair and intracellular signaling, which corresponded to the most abundantly represented functional class of the intermediate level of modulation. Indeed, GADD45A binds to the N-terminal domain of MTK1 [28], a kinase which mediates the activation of both p38 and JNK intracellular signaling pathways in response to environmental stresses. In addition, JNK signaling might also result from the activation of the *MAP4K4* gene (activated 2.5-fold in the microarrays).

Apoptotic genes other than *BAX* and *TNFRSF10B* also included *NRG2.* NRG2 is a member of the epidermal growth factor family, which signals through ErbB family receptors. Because ErbB signals via the MAPK pathway, it represents another level of cross-talk between highly activated functional classes and intracellular signaling. When considering genes at lower levels of modulation, other types of functional profiles were depicted. All the repressed genes were found in these levels of modulation. One-half of these genes were distributed among the same functional classes as the activated genes. However, three categories were exclusively represented by repressed genes, including G-protein-coupled receptor signaling genes, thus confirming the importance of this pathway in the response of hematopoietic cells to irradiation [16].

A recent effort has been made to collect information on radiation-induced genes from different groups [27]. The consistent trend of gene modulation following radiation, whatever the radiation dose and cell type, is the up-regulation of *CDKN1A*, *GADD45*, and *XPC,* which are also activated in CD4+ T lymphocytes (this study). However, in an attempt to highlight the genes that might be regulated by radiation exclusively in CD4+ T lymphocytes, we compared the levels of regulation of the 102 genes specifically identified in this study with those reported by other groups in unsorted cells of lymphocytic origin [7–10]. Being aware of the different array experiments, gene annotation, dose and dose-rate used in these studies, we identified 41 genes in common that had been tested both in our conditions and in unsorted cells by other investigators. Approximately half of these genes were activated in the same direction, although their levels of modulation were more pronounced in CD4+ T cells. Most of them (64%) represented known p53 targets. They included the followings: *ATF3*, *BAX*, *BTG1*, *BTG3*, *CCNG1*, *CDKN1A*, *DDB2*, *FDXR*, *FHL2*, *GADD45A*, *GOLGA4*, *GPX1*, *MLF2*, *MYC*, *ODC1*, *PCNA*, *PRKAB1*, *PTEN*, *PTP4A1*, *RNAH*, *STX6*, *TNFRSF10B*, *TNFRSF6*, *TRIM22*, and *XPC.* The other half of the genes modulated in CD4+ T lymphocytes, of which many are involved in the immune response, are not regulated in the unsorted populations, as reported by other investigators [7–10]. These genes included the followings: *COPE*, *LSP1*, *HLA-A*, *HLA-DQB1*, *ISG20*, *TAX1BP1*, *PCNP*, *INPP5D*, *ATP2A3*, *ATP10D*, *RAB4B*, *RBBP6*, *PAICS*, *KPNB2,* and *SATB1*. This group of genes possibly reflects a modulation that is specific for the CD4+ T cell lineage and that is independent of the p53 pathway.

As the p53 pathway played a predominant role in the radiation response in CD4+ T lymphocytes, the expression of three p53 targets (*DDB2*, *BAX*, and *TNFRSF10B*) selected among two of the highly modulated functional classes (apoptosis and DNA repair) activated in the CD4+ T lymphocytes was tested in different lymphocyte subsets. Globally, the activation levels of the three genes were higher in sorted cells compared to PBLs. Several hypotheses could explain this phenomenon. First, PBLs might contain a minor subpopulation that is far less responsive to irradiation and that would lower the global response of PBLs to irradiation. However, in this study, the tested subpopulations corresponded to 87% (45% of CD4+, 22% of CD8+, 8% of CD19+, and 12% of CD56+) of the PBLs. Another important lymphocyte subgroup constituted by lymphocytic CD4– CD8– $\gamma\delta$+ cells accounted for only 4% of PBLs. Therefore, even if the other unconsidered subpopulations were completely refractory to radiation, they would contribute to only 10–15% of the transcriptional response. Second, antibodies used for

sorting might amplify gene activation. However, this is unlikely, given that the levels of gene expression did not differ significantly between PBLs and any of the subpopulations in the absence of radiation. Third, as a more plausible hypothesis, isolated subpopulations might be more responsive to irradiation than when cultured in the context of PBLs. This difference might be due to the absence of cellular interactions between subpopulations, e.g., through secretion of cytokines, lymphokines, and other soluble signaling molecules.

It has been proposed that different susceptibilities to radiation in cells with a common origin might be linked to differences in the basal level of expression of certain proteins and specifically in the level of the Bcl-2 family proteins [29]. However, no differences in the basal expression levels of the proapoptotic *BAX* and *TNFRSF10B* genes were observed among the cell types. In contrast, their levels of activation induced by radiation differed among the cell subtypes whereas *DDB2* did not. Interestingly, the expression levels of *BAX* were increased in lymphocytes in the following order: NK CD56+ < T CD4+ < T CD8+ < B CD19+, which reflects their differential radiosensitivity to the killing effect of radiation [12, 30–32]. In contrast, the expression level of the other proapoptotic gene *TNFRSF10B* in irradiated cells was not significantly different among the various lymphocyte subpopulations, suggesting that its expression might not be related to the differential radiosensitivity of the cell subtypes. These results suggest that radiation-induced apoptosis in lymphocytes is triggered by at least two p53-dependent pathways. The first one, through BAX, induces cell death by acting on mitochondria, and accounts for the differential radiosensitivity among the cell subpopulations. In the second one, TNFRSF10B mediates apoptosis by acting at the plasma membrane level via interaction with its ligand TRAIL.

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