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Cutting Edge: Inherent and Acquired Resistance to Radiation-Induced Apoptosis in B Cells: A Pivotal Role for STAT3

Dennis C. Otero,* Valeria Poli,† Michael David,* and Robert C. Rickert‡

Radiation-induced apoptosis (RiA) is used therapeutically for tumor cell ablation as well as a tool to characterize hematopoietic cell lineages. We report that the peritoneal B-1 B cell subset is selectively resistant to RiA. Inherent radioresistance is not shared by splenic B-2 or B-1 cells. However, it is conferred upon B-2 cells by BCR crosslinking in the presence of IL-6 or IL-10. In vivo experiments with gene-targeted mice confirm that IL-6 and, to a lesser extent, IL-10 are the relevant stimuli that combine with BCR ligands to promote B-1 cell radioresistance. STAT3 promotes cell survival in response to selected growth factors, and is activated by combined BCR crosslinking and IL-6 (IL-10). Importantly, STAT31+/− B-1 cells become susceptible to irradiation, indicating that STAT3 activation by the BCR in the presence of IL costimuli account for the inherent radioresistance of peritoneal B-1 B cells. The Journal of Immunology, 2006, 177: 6593–6597.

In contrast to mature recirculating B cells (also referred to as conventional or “B-2” cells), the B-1 B cell subset predominates in the peritoneal cavity (PeC) and produces an abundance of protective natural Ab (1). Recent studies have identified CD19+/CD45Rlow/negative progenitor cells in the fetal liver and adult bone marrow as preferentially giving rise to the CD5+ B-1a and CD5− B-1b subsets, respectively (2). This study supports earlier work suggesting that commitment to the B-1 cell compartment (1). This conclusion seems at odds with the general observation that B-1 cells are hyporesponsive to BCR signaling. However, in vitro measurements address acute effects of Ag stimulation, which contrasts with chronic Ag stimulation thought to drive B-1 expansion and maintenance in vivo. Interestingly, peritoneal B-1 cells constitutively express an active form of the STAT3, which is essential for IL-6/IL-10 signaling and serine phosphorylated upon BCR engagement (7, 8). In this study, we report that B-1 cells are selectively resistant to ionizing radiation-induced apoptosis (RiA), and determine the integrated roles of Ag, IL-6/IL-10, and STAT3 in this response.

Materials and Methods

Animals. Mice were obtained from the following sources: wild type (WT), IL-6−/− (B6;129S2-Ile10tm1Kop/J), and IL-10−/− (B6.129P2-Ile10tm1Cgn/J) from The Jackson Laboratory; STAT1−/− (129S6/SvEvTac-Stat1tm1Rds/Tac) from Taconic Farms; STAT5a/b−/− (129-Stat5atm1JniStat5btm2Jni) from Dr. J. Ihle (St. Jude’s Children’s Hospital, Memphis, TN), and VH12 (Tg(Igh-V12CH27/Igh-666−1Sce)) from S. Clarke (University of North Carolina at Chapel Hill, Chapel Hill, NC). Mice lacking STAT3 (129-Stat31−/−) in B cells have been described (9, 10). Animals were housed and handled in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego. For irradiation experiments, mice were exposed to 5–10 Gy gamma irradiation from a cesium source and apoptosis measured in cell populations 24–72 h later.

Cell culture. B cells from 6- to 8-wk-old mice were obtained from the PeC by lavage or single cell suspensions made from spleen. Splenic B-2 cells were purified by negative selection using anti-CD43 beads (Miltenyi Biotec). Cells were cultured at 1 × 106 cells/ml in complete RPMI 1640 and stimulated with indicated concentrations of anti-IgM F(ab′)2 (Jackson Immunoresearch), IL-6 (PeproTech), and IL-10 (PeproTech).

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3 Abbreviations used in this paper: PeC, peritoneal cavity; RiA, radiation-induced apoptosis; HSC, hematopoietic stem cell; PI, propidium iodide; WT, wild type.
Flow cytometry. Single-cell suspensions were prepared and RBC lysed with ACK buffer (0.15 M NH₄Cl, 1 mM KHCO₃, and 0.1 mM Na₂EDTA, pH 7.4). Abs against the following surface markers were obtained from BD Pharmingen: IgD-FITC, IgM-allophycocyanin, CD23-PE, IL6Rx-PE, IL10R-PE, and Annexin V-PE. Apoptotic cells were identified by flow cytometric analysis of sub-G₀/G₁ peaks after staining in 1 mM Tris (pH 8), 0.1% Triton X-100, 0.1% sodium citrate, 0.1 mM EDTA, and 50 μg/ml propidium iodide (PI).

Results and Discussion

B-1 cells are resistant to gamma radiation-induced apoptosis

The use of bone marrow reconstitution for adoptive or autoreconstitution studies is based on the premise that progenitor and mature hematopoietic cells are relatively susceptible to ionizing radiation, whereas non-cycling stem cells are resistant. Similar to hematopoietic stem cells (HSCs), B-1 cells are also known to be long-lived self-renewing cells (1). To determine whether B-1 cells share the property of radioresistance with HSCs, mice were irradiated, and the composition of the B cell compartment was examined by flow cytometry. Importantly, B-1 cells persisted in the PeC for weeks following sublethal (5 Gy) irradiation (Fig. 1, a and b). Bromodeoxyuridine labeling studies confirmed that these cells were not newly generated from the proliferative expansion of a small subset of pre-existing or immigrating B cells (data not shown). By comparison, B-2 cells are eliminated from the PeC as well as the spleen at early time points, but re-emerge after 8 wk as a result of autoreconstitution by bone marrow stem cells (Fig. 1a and data not shown). Lethal (10 Gy) doses of irradiation eliminated all B-2 cells and the vast majority of B-1 cells (Fig. 1b).

Because B-1 cells are not thought to efficiently repopulate from the bone marrow (1). Therefore, we reasoned that most B-1 cells present in the PeC at 8 wk postirradiation were also present before irradiation (Fig. 1a). To directly determine radioresistance of the B cell subsets, purified splenic B-2 cells and peritoneal B-1 cells were exposed to 2 Gy of irradiation and apoptosis tracked over time in vitro. B-2 cells were found to rapidly undergo apoptosis following irradiation, whereas B-1 cells maintained viability as measured by PI, Annexin V, and DiOC₆ staining (Fig. 1c and data not shown). Thus, differentiation into the B-1 subset is associated with acquired radioresistance.

FIGURE 1. Muirine B-1 cells are radioresistant. a, Flow cytometry profiles of peritoneal B-1 (IgM^{high}IgD^{low}) and B-2 (IgM^{low}IgD^{high}) cells at indicated time points postirradiation (5 Gy) (n = 6 mice). b, B cell recovery from PeC 48 h after indicated exposure (n = 3 per group). c, Kinetics of in vitro cell death determined by PI staining after 2 Gy exposure; representative of more than three experiments.

Microenvironment of the peritoneal cavity is critical for B-1 cell radioresistance

B-1 cells can be found in the spleen as well as the PeC and have been shown to differ in their responses to extracellular stimuli based upon anatomic location (6, 11, 12). Characterizing B-1 cells in the spleen is difficult due to their low frequency relative to B-2 cells and phenotypic similarity to marginal zone B cells. Therefore, to investigate whether splenic B-1 cells are similar to peritoneal counterparts in terms of radioresistance, we used mice expressing a rearranged Ig H chain transgene (VH12). This VH₁ rearrangement is expressed in a high frequency of normal peritoneal B-1 cells and, when expressed as a transgene, drives uncommitted B cells into the B-1 cell compartment (5). VH12 mice were exposed to 5 Gy irradiation and the number of B-1 cells in the spleen and PeC were compared relative to non-irradiated mice. Fig. 2a shows that while VH12 peritoneal B cells are radioresistant (like their endogenous counterparts in WT mice), splenic VH12 B-1 cells displayed similar radiation sensitivity to splenic B-2 cells. This finding suggests that the PeC represents a distinct environment that supports the survival of B-1 cells.

Because B-1 cells in the spleen and B-2 cells in the PeC are both susceptible to gamma irradiation, we reasoned that BCR signaling and PeC-associated cytokine/growth factor responsiveness combine to establish the anti-apoptotic program in peritoneal B-1 cells. To test this hypothesis, purified splenic B-2 cells were stimulated with anti-IgM F(ab’)$_2$ and immediately injected i.v. or into the PeC of syngeneic animals. Recipient mice were exposed to 5 Gy of irradiation within 1 h after the transfer and the efficiency of B-2 cell recovery (CFSE-labeled cells) measured at 24 h posttransfer. Fig. 2b shows that BCR-stimulation conferred survival to B-2 cells transferred into the PeC but not the spleen of irradiated recipients. No evidence of emigration from the PeC to the blood or secondary lymphoid tissues was detected following irradiation (data not shown). In addition, proliferative expansion cannot account for this effect since significant partitioning of the CFSE membrane dye was not observed (data not shown).

Whereas BCR engagement provided a significant survival advantage to B cells transferred into the PeC (Fig. 2b), we found that BCR stimulation in vitro offered minimal protection from
and activated B-2 cells (Fig. 2). IL-6R2c). Despite its well-established role as a B cell growth and survival factor, IL-6 alone did not protect from RiA (Fig. 2e). Aortic arches of peritoneal and splenic (from VH12 transgenic mice). Relative numbers of B-1 (CD23negIgMhigh) and B-2 (CD23posIgMlow) cells were determined 48 h postirradiation. Both IL-6 and IL-10 in particular, in promoting B-1 cell protection from RiA.

**STAT3 activation is necessary for B-1 cell radioresistance**

IL-10 signals through STAT1, STAT5, and STAT3, whereas IL-6 primarily activates STAT3. B-1 cells in the PeC express constitutively phosphorylated STAT3 and STAT1 (13), presumably due to stimulation by cytokines present in the PeC. It has been reported that anti-Ig and IL-6/IL-10 synergize in the activation of STAT3 (14), consistent with our hypothesis that BCR engagement in the presence of IL-6/IL-10 protects B cells from RiA via activation of STAT3. To directly determine whether this is the case, we ablated STAT3 in the B lineage by crossing mice bearing a loxP-flanked Stat3 gene with mice expressing the Cre recombinase gene under the control of the B cell-specific Cd19 promoter (9, 10). Importantly, B-1 cells were

**FIGURE 2.** Cytokines and cell activation contribute to B cell radioresistance. **a,** Recovery of splenic B-1 cells from VH12 transgenic mice 48 h postirradiation (5 Gy, n = 3 mice). **b,** Rescue of B-2 cells from RiA. Purified CFSE-labeled splenic B-2 cells were left untreated or stimulated with anti-IgM (10 μg/ml) and immediately injected i.p. or i.v. Recipient mice were irradiated (5 Gy) and transferred B cells from PeC or spleen were enumerated 24 h postirradiation. Graph represents percent cell recovery from irradiated mice compared with non-irradiated mice (n = 3 mice per group). **c,** In vitro rescue of B cells from RiA. Purified splenic B cells were irradiated (2 Gy) following an 18-h incubation with anti-IgM and/or IL-6. Apoptosis was measured by PI (sub-G0-G1) staining 24 h postirradiation; representative of four experiments. **d,** Expression levels of IL-6 and IL-10 receptor on ex vivo and stimulated (24 h) B cells; LPS (1 μg/ml), anti-CD40 (10 μg/ml), and anti-IgM (1 μg/ml). **e,** B cell recovery from the PeC of IL-6 /IL-10 / mice. Relative numbers of B-1 (CD23negIgMhigh) and B-2 (CD23posIgMlow) cells were analyzed before and after (48 h) irradiation (5 Gy). Graph represents percent cell recovery from the PeC of irradiated mice compared with non-irradiated mice (average ± SEM of at least three mice per group).
maintained in the PeC of conditional STAT3 \(-/-\) mice, albeit at a somewhat lower frequency, but were significantly more susceptible to RiA than strain/age-matched WT counterparts (Fig. 3a).

In addition to homodimers, STAT3 can form heterodimers with STAT1, and to a lesser extent with STAT5. Therefore, to assess the specificity of the STAT3 defect in RiA, the B-1 cell compartment was also examined in STAT1 \(-/-\) and STAT5a/b \(-/-\) mice. Unlike conditional STAT3 \(-/-\) mice, both STAT1 \(-/-\) and STAT5a/b \(-/-\) mice showed significantly reduced numbers of peritoneal B cells (Fig. 3a). The B cell defect in STAT5a/b \(-/-\) mice is likely attributed to impaired IL-7R signaling in early B cell development (15, 16), whereas a B-1 cell defect has not been previously noted in STAT1 \(-/-\) mice. Nonetheless, it appears that neither STAT1 nor STAT5a/b has a clear role in B-1 cell RiA (Fig. 3a), illustrating that this is a unique property of STAT3.

We have shown that costimulation of splenic B-2 cells with anti-IgM and IL-6/IL-10 confers radioresistance (Fig. 2). To determine whether this acquired property is STAT3-dependent, B-2 cells from WT and STAT3 \(-/-\) mice were cultured overnight in the presence of IL-6/IL-10 and/or anti-IgM, irradiated (2 Gy) and viability assessed 24 h postirradiation (Fig. 3b). Both IL-6 and IL-10 were found to synergize with anti-IgM treatment in protecting B-2 cells from RiA. This effect was largely dependent on STAT3, which was not detectable in splenic B-2 cells from STAT3 \(-/-\) mice (data not shown). Therefore, STAT3 appears to actively repress RiA downstream of the BCR and the IL-6R or IL-10R complexes.

Our data demonstrate that B-1 cell radioresistance is a property of B-1 cells conferred by Ag recognition in the context of the cytokine microenvironment of the PeC. BCR costimulating induces up-regulation of IL-6Rα, allowing for synergistic activation of STAT3 by the BCR and IL-6R. IL-6 and IL-10 activate STAT3 via the Jak kinases, which phosphorylate STAT3 at Tyr705, resulting in dimerization and nuclear translocation (7). We previously found that BCR engagement selectively induces STAT3 Ser727 phosphorylation in the absence of Tyr705 phosphorylation (8). This modification augments transcriptional activation of STAT3 in the context of Tyr705 phosphorylation (17). In its homodimeric form, STAT3 has oncogenic potential (18, 19), likely through the up-regulation of target pro-survival genes such as bcl-2, bcl-xL, and ncl-1, or down-regulation of fas. Thus, B cell malignancies should be considered for emerging clinical applications for STAT3 inhibitors.

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**Disclosures**

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