Purinergic P2Y\textsubscript{14} receptor modulates stress-induced hematopoietic stem/progenitor cell senescence

Joonseok Cho,1,2 Rushdia Yusuf,3,4,5 Sungho Kook,1,2,6 Eyal Attar,3,4,5 Dongjun Lee,3,4,5 Baehang Park,1,2 Tao Cheng,7 David T. Scadden,3,4,5 and Byeong Chel Lee1,2

Introduction
Organisms inevitably encounter a variety of stresses during their lifetimes, including radiation, oxidation, and infection. The nature and efficiency of the response to stress is a fundamental determinant of an organism's fitness, with dysfunctional responses serving as putative instigators of malignancy and degenerative diseases.

Nucleotides, long known as metabolic substrates, are now also recognized as key extracellular messengers that regulate diverse aspects of homeostasis in various pathophysiological conditions (1). Stress causes purines and pyrimidines to accumulate in the extracellular space, which alerts the cell to danger through interaction with purinergic receptors (2). They have been shown to serve as a "find me signal" for macrophages to detect and engulf apoptotic cells (3).

Purinergic receptors are classified into P1 and P2 receptors, based on their ligand binding and function (4). P2 receptors are further subdivided into the P2X (ion channel) and the P2Y (G protein coupled) receptor subtypes. P2 receptors are detected not only in mammalian species, but also in chicken (5) and Xenopus (6). The homology between P2 receptors in the amino acid sequence is relatively low (19%–55% sequence identity at the amino acid level) (7, 8).

The role of P2 receptors as regulators of hematopoiesis has been documented (9, 10), but the underlying mechanisms by which purinergic receptors exert their effects in hematopoietic cells have not been studied in detail. Hematopoietic tissues are among the most sensitive to ionizing radiation–induced (IR-induced) damage. While IR can result in either apoptosis or senescence, it has been suggested by some that stress-induced premature senescence (SIPS) may predominate over apoptosis (11, 12). It has also been reported that IR selectively induces senescence in HSCs (13). HSC senescence represents an irreversible loss of proliferation capacity and could compromise HSC ability to react to environmental stress to maintain their delicate homeostatic balance. How stem cells respond or adapt to stress has central implications for regenerative medicine.

We previously constructed a subtractive cDNA library to enrich for differentially expressed transcripts from adult human BM-derived hematopoietic stem progenitor cell (HSPC) populations (G0, CD34\textsuperscript{+}CD38\textsuperscript{−}) (14). Among the genes isolated from the subtractive cDNA library, P2RY14, a 7-transmembrane G protein–coupled purinergic receptor (7TM GPCR), shows highly restrictive expression in human HSPC, and its expression is associated with cell-cycle dormancy.

Herein, we provide evidence demonstrating that P2Y\textsubscript{14} expression is indispensable for maintaining homeostasis in HSPCs and embryos under stress and define it as a receptor mediator of cell senescence.
P2Y14 deficiency increases the susceptibility of HSPCs to radiation stress. (A) Q-PCR analysis of P2ry14 mRNA: mRNA from BM cells bearing the indicated phenotype was analyzed by Q-PCR. The expression was normalized to GAPDH. The expression level in lineage positive (Lin⁺) cells was arbitrarily set to 1. Q-PCR was done in duplicate. B, B cells (B220⁺); T, T cells (CD3⁺); mono, monocytes (CD11b⁺). (B) Cells were gated as indicated, and the expression of P2Y14 was measured within the gates. The percentage of P2Y14⁺ expressing (P2Y14⁺) cells in indicated compartments is plotted on the y axis. The data are representative of at least 3 independent experiments, each with 3 mice per group. (C) Mice of the indicated genotypes were exposed to TBI (3 × 5 Gy). Recipients were allowed to recover for 15 days before the next dose was administered. The number of BM cells was counted within the marrow of femur and tibia. (D) The number of LSK cells was measured after TBI (3 × 5 Gy TBI, 15 days apart). Data show representative mice of at least 6 animals analyzed per group. Statistical analyses were carried out using 1-tailed Student’s t test (C and D) and 2-tailed Student’s t test (B). *P < 0.05; **P < 0.01.

**Figure 1**

P2Y14 deficiency renders HSPCs more susceptible to radiation-induced senescence and cell death. Mice deficient in P2ry14 were generated by the targeted gene deletion of the sequences encoding TM2–TM7 as described (15). Absence of P2Y14 in KO (P2ry14−/−) mice was confirmed by genotyping, quantitative RT-PCR (Q–RT-PCR), and flow cytometry analysis (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI61636DS1). P2Y14 KO mice displayed no obvious phenotypic abnormality when compared with their heterozygous (HT) (P2ry14+/−) and WT (P2ry14⁺/⁺) littermates. Breeding of P2Y14 HT mice resulted in approximately the expected Mendelian ratios of offspring, indicating that the mutation is not associated with embryonic or neonatal lethality (16). Medians of wbc count, rbc count, platelet (PLT) count, and hemoglobin (HGB) count were comparable between P2Y14 KO and WT mice (Supplemental Figure 2). Similarly, BM common lymphoid progenitors (CLPs), common myeloid progenitors (CMPs), granulocyte-monoocyte progenitors (GMPs), and megakaryocyte-erythrocyte progenitors (MEPs) were not significantly different (data not shown). Modest increases in multipotent progenitors (CD34⁺, Lin⁻, Sca-1⁻, c-Kit⁻) (CD34⁻-LSK) (1.8- to 2.5-fold, P = 0.04) and LSK (−1.3 fold, P = 0.006), but no statistically significant changes in CD150⁺CD48⁻ LSK cells (P = 0.17) were observed in KO compared with WT littermates (Supplemental Figure 3). Thus, P2Y14 KO mice have seemingly normal hematopoiesis under steady state conditions.

P2ry14 is detected in various types of hematopoietic cells. However, P2ry14 expression is particularly prominent in murine LSK cells (Figure 1A), consistent with our prior findings in the human HSPCs (14). Thus, the expression of P2ry14 preferentially occurs in HSPCs in both mice and humans.

We next assessed the percentage of P2Y14⁺ expressing cells (P2Y14⁺) in previously defined HSPC populations. The specificity of the P2Y14 antibody was confirmed by the lack of P2Y14 expression in P2Y14 KO Lin⁻ cells (Supplemental Figure 1). Enriching primitive HSCs using previously known phenotypic markers (e.g., CD34⁻-LSK or CD150⁺CD48⁻ LSK) increased the percentage of P2y14 expressing cells (Figure 1B). However, P2Y14⁻ cells represented a relatively small proportion of cells within previously defined HSCs, raising the possibility that P2Y14 expression may define a functionally unique HSPC subset.

Purinergic receptors, including P2Y14, are hypothesized to play a role in modulating the stress response. Evaluation of P2Y14 expression demonstrated increased levels in hematopoietic cells in association with various types of stress. (Supplemental Figure 4). These data reinforce the potential link of P2Y14 to a stress response (17), which we investigated further.

Hematopoietic cells are extremely vulnerable to genotoxic stress-induced cell injury. We therefore assessed whether P2Y14 deficiency modulates the susceptibility of hematopoietic cells to radiation stress. Since different doses of IR can produce different biological consequences, we examined the effects of low (3 Gy), medium (6 Gy), and high (8 Gy) doses of radiation on the IR-induced cell injury. 3 Gy total body irradiation (TBI) did not cause a statistically significant difference in IR-induced cell death between WT and KO LSK cells (Supplemental Figure 5). At doses of greater than 8 Gy,
Figure 2
P2Y14 deficiency increases the susceptibility of HSPCs to radiation-induced senescence and cell death. (A) Mice of the indicated genotypes were exposed to TBI (6 Gy). SA–β-gal activity was determined using C12FDG. Percentage of SA–β-gal–positive LSK population (upper) is expressed as mean ± SD. The data are representative of 6 mice per group. Representative histograms of SA–β-gal staining in gated LSK cell (lower). (B) BM cells from irradiated P2ry14−/− and WT mice (6 Gy TBI) were stained with the indicated antibodies. Lineage-committed progenitor cells were gated based on defined phenotypic criteria, and cellular senescence was measured in each gated population. Numbers indicate the percentage of C12FDG+ cells in the indicated gates. The accompanying graph shows the mean percentage of C12FDG+ cells (± SD) in each gated population (n = 5/genotype). (C) Cell death analysis in gated LSK cells. Cell death was measured by quantification of annexin V+ or DAPI+ (gated on annexin V−) cells 8 hours after TBI (6 Gy). The data are representative of 2 independent experiments each, with BM cells pooled from 2 mice per group. NAC (100 mg/kg) was injected s.c. 4 hours before and 2 hours after TBI. Representative flow cytometric analysis of annexin V− (upper left) and DAPI−, annexin V− (lower left) LSK cells is shown. Percentages of gated cell populations are indicated. Statistical analyses were carried out using 1-tailed Student’s t test (C) and 2-tailed Student’s t test (A and B). *P < 0.05; **P < 0.01.
only a very small number of HSPCs were alive (data not shown), making further analysis difficult. At 5 to 6 Gy TBI, a distinct effect on P2Y14-deficient LSKs compared with WT counterparts was noted (Supplemental Figure 5). Others have documented that TBI at those doses rarely resulted in animal death, but induced both acute and long-term hematopoietic dysfunction (13).

On the basis of these results, P2Y14 KO and WT littermate mice were exposed to 5 Gy TBI, and the number of nucleated cells in the BM was measured. As expected, WT mice showed a reduction of BM cell numbers in response to radiation. However, a more severe reduction was observed in P2Y14 KO mice (Figure 1C). We then examined whether P2Y14 expression in stem/progenitor cells played a role in modulating their susceptibility to radiation. As shown in Figure 1D, P2Y14 KO LSK cells were more susceptible to IR stress than WT counterparts and showed a considerably lower LSK cell number (WT vs. KO: 1.92% ± 0.36% vs. 1.08% ± 0.55%, P = 0.037). Preferential decrease of the P2Y14 KO LSK cells could be the result of increased susceptibility to IR-induced senescence or cell death or both. Since sublethal radiation has been reported to selectively induce HSC senescence (13), we focused on this cell process. LSK cells lacking P2Y14 showed a markedly increased level of senescence-associated β-gal (SA–β-gal) activity (P = 0.003) (Figure 2A) consistent with increased susceptibility to IR-induced senescence. Differential sensitivity to IR-induced senescence was observed in multipotent as well as lineage-committed progenitor cells lacking P2Y14, including CLP, CMP, GMP, and MEP (Figure 2B). Radiation induces both apoptotic and nonapoptotic cell death (18). There was a modest trend toward increased IR-induced apoptosis (annexin V+) in P2Y14 KO LSK cells (Figure 2C). However, a higher rate of annexin V–, DAPI+ cells (P = 0.047) was detected (Figure 2C), suggesting that P2Y14 KO HSPCs may die primarily through nonapoptotic rather than apoptotic pathways.

Figure 3
P2Y14 deficiency increases the susceptibility of HSPCs to various hematological stresses. (A) A mixture of BM cells from P2ry14−/− (CD45.2) and congenic WT (CD45.1) mice were transplanted into recipients (CD45.1.2, n = 16) as described in Methods. The x axis denotes the number of weeks after transplantation. Upper panel: untreated mice (n = 6); lower panel; 5 weeks after transplantation, a group of recipients (n = 10) were irradiated (6 Gy TBI) and then the contributions of WT (CD45.1) and P2ry14−/− (CD45.2) cells to the recipients' blood were measured. Lightning bolts mark the timing of IR. (B) BM cells from P2ry14−/− and WT mice were transplanted as described in A. Eight to nine months after transplantation, recipients (n = 8) were irradiated (6 Gy) and the frequency of WT and P2ry14−/− derived LSK cells in recipients' BM was assessed at 5 weeks after IR (right). The accompanying graph (left) shows the absolute numbers of LSK cells. (C) WT and P2ry14−/− BM cells were transplanted as described in A. Five weeks after transplantation, recipients (n = 6) were treated with 5-FU (150 mg/kg). Recipients were allowed to recover for 9 days before the next dose was administered. The x axis denotes the number of weeks after transplantation. Arrows mark the timing of injection. Ratio of WT and P2ry14−/− cells in recipients' blood was determined 7 days after each 5-FU injection. (D) Contribution of WT and P2ry14−/− cells after secondary transplantation (n = 4/genotype). The x axis denotes the number of weeks after transplantation. Statistical analyses were carried out using 1-tailed Student's t test (B) and 2-tailed Student's t test (A, C, and D). *P < 0.05; **P < 0.01; ***P < 0.001.
P2Y14 deficiency compromises the ability of HSPCs to respond to hematological stress. To assess whether WT-derived cells have a competitive advantage over P2Y14 KO–derived cells under stress, we performed competitive repopulation assays. P2Y14 mice were backcrossed 8 times with C57BL/6J (B6) mice, which made them more than 99% genetically identical to B6 mice. Therefore, BM cells from CD45.1 congenic strains of B6 were used as WT control (B6, CD45.1). When P2Y14 KO (B6, CD45.2) and WT BM (B6, CD45.1) cells were cotransplanted into lethally irradiated WT recipients (CD45.1.2), there was a similar contribution from KO and WT cells in recipient mice 5 weeks after transplantation, and this ratio remained constant for at least 1 year (Figure 3A). However, when these recipient animals were irradiated, P2Y14 KO–derived cells (CD45.2) showed a progressive decline in their competitive capacity against WT-derived cells (CD45.1) (Figure 3A). These changes became more prominent as the recipients were treated with a second round of IR (Figure 3A).

We then asked whether this preferential reduction of P2Y14 KO–derived cells within the WT microenvironment was due to higher sensitivity of P2Y14 KO HSPCs to radiation. Recipient mice undergoing IR treatment showed a marked difference in the frequency of donor-derived LSK cells in their BM, displaying a preferential depletion of P2Y14 KO–derived LSK cells (KO-derived LSK vs. WT-derived LSK; 27% vs. 69%) (Figure 3B). These data suggest a hematopoietic cell-autonomous basis for the differential sensitivity to IR.

Multiple doses of cytotoxic chemotherapy are generally used in cancer patients, and we evaluated whether P2Y14 expression affected resistance to repetitive 5-fluorouracil (5-FU) treatment. Recipient animals were treated with 5-FU and allowed to recover for 9 days before the next dose was administered. The degree of contribution by P2Y14 KO– and WT-derived cells was approximately equal until the sixth injection (Figure 3C). However, the level of P2Y14 KO–derived cells was dramatically reduced after the seventh injection and remained low thereafter (Figure 3C).

If the observed phenotypic changes in P2Y14 KO mice are due to increased susceptibility of P2Y14 KO HSPCs to stress, one would expect that P2Y14 KO HSPCs would display a repopulating disadvantage in the setting of serial transplantation, since this procedure also imposes extreme stress on the HSCs (19). In the first round of transplantation, equal contributions from the WT versus P2Y14 KO cells were observed in recipients’ peripheral blood (PB), similar to that shown in Figure 3A. However, WT cells began to show a competitive repopulating advantage over P2Y14 KO cells when they were subjected to secondary transplantation (Figure 3D).

Taken together, these results indicate that P2Y14 KO HSPCs are functionally compromised in their ability to cope with multiple types of hematological stress.

P2Y14 deficiency renders HSPCs more susceptible to senescence during aging. P2Y14 KO mice showed no apparent outward signs of accelerated aging at up to 2 years of age (data not shown). Unless the mice were exposed to stress, no obvious differences in senescence and cell death were observed between the P2Y14 KO and WT HSPCs at a relatively young age (>4–6 months old, see Figure 2). However, P2Y14-deficient LSK (Figure 4A) and CD150+CD48– LSK (Figure 4B)
cells displayed significantly higher levels of SA–β-gal activity compared with WT counterparts at 2 years of age. Differences were even more obvious when the BM cells from the old mice were further transplanted into recipient animals (Figure 4, A and B). Collectively, these findings suggest the possibility that P2Y14 deficiency may also affect HSPC senescence in the process of aging.

**P2Y14 is implicated in genotoxic stress responses during embryonic development.** Many purinergic receptors are abundantly expressed during embryonic development (20). P2Y14 is also expressed at a high level in the yolk sac and aorta-gonad-mesonephros (AGM) at E10.5 (our unpublished observations). The developing embryo is exceptionally sensitive to damage from radiation, and we thus sought to determine whether P2Y14 deficiency affects the embryo’s ability to cope with radiation stress. Pregnant females (HT mating) were exposed to 1.5 Gy TBI because doses higher than 1.9 Gy dramatically increased fetal death, as we and others observed (21, 22). On the 18th to 19th day of gestation, pregnant dams were autopsied and fetuses were examined for external abnormalities. Exposing pregnant dams to 1.5 Gy TBI did not result in a significant prenatal death or teratogenic malformation, and the genotype ratio of viable embryos was close to the expected Mendelian ratio. These data suggest that P2Y14 deficiency did not cause preferential post-implantation loss of P2Y14 KO embryos, at least at this dose of radiation. Of note, however, following prenatal radiation exposure, mean body weights of P2Y14 KO embryos were significantly lower ($P = 0.02$) than their HT or WT littermates (Figure 5A). The weight

![Figure 5](https://doi.org/10.1172/JCI61636)
loss in P2Y14 KO embryos following irradiation (IR) can be potentially linked to a differential susceptibility of P2Y14 KO embryos to IR-induced premature senescence (23, 24). We therefore determined whether P2Y14 KO embryos are more susceptible than their HT or WT littermates to IR-induced senescence. The level of SA–β-gal was preferentially and markedly elevated in P2Y14 KO embryos upon IR (Figure 5B), suggesting that deletion of P2Y14 sensitized embryos to IR-induced senescence.

We further assessed senescence induction using mouse embryonic fibroblasts (MEFs) that we also noted abundantly express P2ry14 mRNA (Supplemental Figure 6). MEFs were subjected to serial passages under standard culture conditions, which supply supraphysiological levels of oxygen (20% O2). It was reported that this high oxygen level imposes a state of oxidative stress on MEFs, resulting in DNA damage (25). P2Y14 KO MEFs exhibited slow proliferation compared with WT MEFs and reached a growth plateau earlier as compared with WT MEFs (Figure 5C). P2Y14 deficiency accelerated the rate of senescence in MEFs upon serial passaging, as determined by SA–β-gal staining and typical senescent morphology of large and flat cells (Figure 5D). The senescent phenotype of P2Y14 KO MEF also became apparent following IR treatment when it was compared with that of WT counterparts (Supplemental Figure 7). Taken together, these findings indicate that the senescence-prone phenotype induced by P2Y14 deficiency could be observed at both a cellular and whole organism level and is not restricted to hematopoietic cells.

Elucidation of molecular mechanisms underlying the hypersusceptibility of the P2Y14 deficient HSPCs to radiation-induced senescence. The forma-
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Figure 7
Impact of P2Y14 deficiency on senescence-associated molecules. (A) WT and P2ry14−/− BM cells were transplanted as described. Eight months after transplantation, recipient mice were either left untreated (n = 4, left) or irradiated (n = 4, right) with TBI (6 Gy). LSK cells derived from WT (CD45.1) or P2y14−/− (CD45.2) donors in the recipients were sorted and subjected to Q-PCR analysis, respectively. The expression level in WT cells was arbitrarily set to 1. The fold change in expression of each gene was calculated using the ΔΔCt method. The expression was normalized to GAPDH. The 2-tailed Student’s t-test was used. (B) Four weeks after TBI (6 Gy), LSK and CD150+CD48− LSK cells were gated and analyzed for the expression of p16ink4a by flow cytometry analysis. Mice were analyzed individually (n > 3 mice/group). Representative flow cytometric analysis of p16ink4a in gated LSK and CD150+CD48 LSK cells is shown (left). The 2-tailed Student’s t-test was used. (C) Western blot analysis of WT and P2y14−/− MEFs: early passage WT or P2ry14−/− MEF cells were prepared and serially passaged following a 3T3 protocol. Cell lysates were probed with the indicated antibodies. Autoradiographs were analyzed by densitometry. The intensity observed in passage no. 2 WT MEF cells was normalized to the β-actin and arbitrarily set to 1.0. The normalized signal intensities of ppRb and pRb proteins were expressed as a ppRb/pRb ratio. The ratio of ppRb/pRb in passage no. 2 WT MEF cells was arbitrarily set to 1.0. Number denotes passage numbers. *P < 0.05; **P < 0.01.

When cellular senescence is induced, there are typical expression patterns of the cell-cycle regulators (30), including the cyclin-dependent kinase inhibitor p16INK4a (31).

We did not detect differences in expression levels of p16INK4a transcripts in freshly prepared P2Y14 KO and WT LSK cells (data not shown). When KO cells were transplanted into primary recipients, p16INK4a expression began to increase in P2Y14 KO LSK cells (a moderate 1.9-fold increase compared with that in WT LSK) (P = 0.003) (Figure 7A). When these primary recipients were subjected to subsequent radiation, P2Y14 KO LSK cells displayed a marked increase in p16INK4a expression (6- to 8-fold, P = 0.006) (Figure 7A). The observed changes in p16INK4a transcripts were paralleled by changes in p16INK4a protein in LSK and CD150+CD48− LSK cells, detected by flow cytometric analysis (Figure 7B).

To further determine the nature of premature senescence in P2Y14 KO cells at the protein level, MEF cells were analyzed for expression of senescence-associated proteins. With higher passage numbers, elevated levels of p16INK4a protein were observed in P2Y14 KO MEFs (Figure 7C). The retinoblastoma protein (pRb) is hypophosphorylated in senescent cells, and its function is necessary for inducing the senescent phenotype (32, 33). It is also reported that the increased expression of p16INK4a results in the accumulation of hypophosphorylated pRb (34). In line with these findings, hyperphosphorylated forms of RB (ppRb) became significantly lower in P2Y14 KO MEFs at passage 4 (see the ratio of ppRb to pRb in Figure 7C).

Hyperradiosensitivity of P2Y14 KO HSPCs may relate to their differential DNA repair capacity. However, similar levels of DNA damage (at 30 minutes after IR) and repair (at 6 hours after IR), as mea-
sured through the γ-H2AX assay, were detected in P2Y14 KO HSPCs in comparison with WT counterparts (Supplemental Figure 8), arguing against an altered response or ability to repair DNA double-strand breaks. There were no cell-cycle differences detected between WT and KO LSKs either before or after IR (Supplemental Figure 9).

p38 MAPK has been shown to act downstream of ROS, and its activation in HSC can lead to a defect in HSCs (26, 27). It is also a known mediator of the cytokine release phenomena associated with senescence (35). We thus determined whether P2Y14 KO HSPCs display differential activation of p38 MAPK under radiation...

**Figure 8**
Analysis of molecular pathways underlying the increased susceptibility of P2Y14-deficient HSPCs to radiation. (A) WT (red lines) and P2y14−/− (blue lines) mice were exposed to TBI (6 Gy). Mice (n > 4 mice/genotype) were sacrificed immediately after IR and their LSK (upper) and CD150−CD48− LSK (lower) cells were analyzed for phosphorylated p38 MAPK by flow cytometry. NAC was treated as described. The accompanying graphs (right) show the MFI of phospho–p38 MAPK in LSK (upper) and CD150−CD48− LSK (lower) cells. (B and C) Irradiated WT and KO mice (6 Gy) were sacrificed 8 hours after IR and their LSK and CD150−CD48− LSK cells were analyzed for cell death. Representative dot plots and histograms are shown in Supplemental Figure 10. p38 MAPK inhibitor (SB202190) and JNK inhibitor (SP600125) were administered 30 minutes before and immediately after TBI. (C) Clonogenic capacity of the treated cells was analyzed by CFU assay. (D) P2Y14 KO mice were exposed to TBI (6 Gy). SB202190 and SP600125 were administered as described above. Mice were sacrificed 30 minutes after IR, and their BM cells were transplanted into recipient mice (n > 5/each group). LSK cells were analyzed at 4 to 5 weeks after transplantation for C12FDG expression. *P values in Figure 8 were determined using 2-tailed Student’s t test. *P < 0.05; **P < 0.01.
radiation stress. Since ROS generation occurs within seconds of radiation exposure and persists for 2 to 5 minutes after IR (36), the activation of p38 MAPK was analyzed at an early time point immediately after TBI. While there was no detectable difference in p38 MAPK activity between WT and KO HSPCs under homeostatic conditions, p38 MAPK activity was notably higher in P2Y14-deficient LSK and CD150<sup>+</sup>CD48<sup>−</sup> LSK cells following radiation (Figure 8A). Of note, p38 MAPK activation was transient, as its level returned to near basal control levels about 5 hours after TBI (data not shown). To investigate whether this aberrant activation of the p38 MAPK in P2Y14-deficient HSPCs accounts for their increased susceptibility to IR, a specific p38 MAPK inhibitor was administered to animals. p38 MAPK inhibitor treatment not only reduced the susceptibility of P2Y14-deficient LSK and CD150<sup>+</sup>CD48<sup>−</sup> LSK cells to IR-induced cell death (Figure 8B and Supplemental Figure 10), but also restored their clonogenic capacity (Figure 8C). Furthermore, p38 MAPK inhibition significantly ameliorated the susceptibility of P2Y14 KO HSPCs to IR-induced senesence (Figure 8D). Of note, NAC treatment almost completely abolished the p38 MAPK activation in HSPCs (Figure 8A). Both p38 MAPK and c-JNK are known to be activated by IR and share many common signaling components (37). However, p38 MAPK and JNK can mediate distinct stress responses depending on the nature and duration of the stress and the cellular context (38). The inhibition of JNK with its inhibitor, SP600125, did not exert any measurable effect on clonogenic capacity (Figure 8C), but was able to reduce IR-induced LSK cell senescence (Figure 8D). These data are consistent with P2Y<sub>14</sub> limiting radiation-induced activation of p38 MAPK and JNK, perhaps by reducing ROS.

While the signaling pathways triggered by the P2Y<sub>14</sub> receptor are still poorly documented, it is reported that P2Y<sub>14</sub> receptor exerts its effects primarily via the Gi/o family of the heterotrimeric G protein family (39, 40). To determine whether the increased sensitivity of P2Y<sub>14</sub> KO HSPCs to IR is mediated via the P2Y<sub>14</sub> receptor acting through Gi/o, P2Y<sub>14</sub> KO and WT mice were injected with pertussis toxin (PTX) and then exposed to 6 Gy TBI. BM cells (CD45.2) were harvested and subsequently transplanted into recipient animals (CD45.1.2). We then examined the effects of PTX treatment on IR-induced HSPC senescence. Since PTX specifically inactivates Gi/o-mediated signaling pathways (39), one would expect that interruption of Gi/o signaling in WT cells by PTX would recapitulate the phenotype of KO HSPCs in response to IR-induced senescence, if the observed difference is mediated via P2Y<sub>14</sub> receptor. As shown in Figure 9A, upon PTX treatment, WT cells demonstrated senescence induction similar to that of KO cells. Also, the difference between WT and KO LSK cells was abrogated, with PTX supporting the notion that P2Y<sub>14</sub> signals through Gi/o to modulate IR-induced LSK cell senescence.

Taken together, our data support the hypothesis that the Gi/o-coupled P2Y<sub>14</sub> signaling axis modulates stress-induced HSPC senescence, at least in part, through a mechanism involving ROS, p38 MAPK/JNK, and p16/Rb (Figure 9B).

**Discussion**

Stem cells possess the capacity to replenish damaged or dysfunctional cells throughout life. However, stem cells are themselves vulnerable to stress-mediated cellular injury, and a progressive diminution of stem cells in response to stress leads to loss of tissue homeostasis. Therefore, preserving the integrity of HSC function is closely associated with morbidity and mortality in patients receiving radiation and chemotherapy. Despite these significant clinical implications, the molecular machinery of how stem cells cope and adapt to the various types of stress is poorly defined.

In this study, we demonstrate that a purinergic receptor, P2Y<sub>14</sub>, plays an indispensable role in countering the deleterious effects of multiple types of cell stress in HSPCs.

Under physiological conditions, P2Y<sub>14</sub> appears to not be essential for normal embryonic development nor for the maintenance of tissue homeostasis in the adult organism. However, the embryo is exceptionally sensitive to radiation-induced damage, and under the stress conditions of TBI, P2Y<sub>14</sub> KO embryos were more prone to undergoing IR-induced senescence than WT embryos. In the adult, hematopoietic cells are among the most sensitive to radiation injury. P2Y<sub>14</sub> KO mice were more sensitive to radiation,
showing a more severe reduction in the number of BM cells than that observed in WT mice. This was accompanied by a more profound reduction of LSK cells in P2Y_{14} KO mice, suggesting that loss of P2Y_{14} confers increased susceptibility to radiation in HSPC populations. P2Y_{14} KO mice were also hypersensitive to other hematologic stress. The response of P2Y_{14} KO mice to s-FU treatment documented a higher susceptibility of P2Y_{14} KO HSPCs to repetitive myelotoxic stress. Furthermore, P2Y_{14} KO BM cells were out-competed by WT BM cells in secondary recipient animals. Taken together, these results suggest that P2Y_{14} KO HSPCs are functionally compromised in their ability to cope with hematologic stress.

A sublethal dose of TBI induces HSC senescence (13), and P2Y_{14} KO HSPCs showed typical phenotypic and molecular changes associated with cellular senescence upon IR, suggesting that the differential response of P2Y_{14} KO cells to IR may be due to their increased sensitivity to IR-induced senescence. However, senescent cells eventually undergo apoptosis, so that the accumulation of senescent cells can also lead to increased apoptosis in P2Y_{14} KO mice. While P2Y_{14} is expressed in a relatively small fraction of LSK and CD150^{+}CD48^{−} LSK cells (Figure 1B), the difference in IR-induced senescence phenotype was detectable in a larger fraction of HSPCs. One potential explanation for this might be due to the increased expression of P2Y14 in a subset of irradiated HSPCs. Indeed, as demonstrated by previous reports (17, 21) and our results here (Supplemental Figure 4), IR and other stress cause the increase of P2Y_{14} expression in stressed cells. It may also be possible that our antibody-based criteria for defining P2Y_{14}-expressing cells in Figure 1B underestimate the fraction of cells with a functionally meaningful level of the protein.

Radiation induces cellular injury mainly through the generation of ROS, which is a common mediator of cellular senescence (41). HSCs are much more sensitive to oxidative stress than the progenitors (26), and excessive levels of ROS are especially detrimental to HSCs. The functional defects of HSCs observed in ATM^{−}/Bmi-1^{−}, and Foxo-deficient mice are all attributed to ROS-induced oxidative damage (26, 27, 42), underscoring the crucial role of ROS in stem cell injury. A higher level of mitochondrial ROS was detected in P2Y_{14} KO LSK cells than in WT counterparts following IR. Consequently, it caused a significant loss of ∆ψm in P2Y_{14} KO LSK cells. Meanwhile, the antioxidant NAC markedly attenuated IR-induced HSPC depletion and senescence in P2Y_{14} KO mice, suggesting that the increased sensitivity of P2Y_{14} KO cells to IR stress is likely attributable, at least in part, to dysregulation of ROS in P2Y_{14} KO cells following TBI.

There was a differential activation of p38 MAPK between P2Y_{14} KO and WT LSK cells following IR. Since p38 MAPK activation in HSC can lead to a defect in HSCs (26, 27), ROS-induced hyper-activation of p38 MAPK in P2Y_{14}-deficient HSPCs is a potential mechanism underlying the increased susceptibility to IR stress. While we were unable to detect JNK activation in HSPC cells due to the lack of specific antibody that works for flow cytometry analysis, JNK inhibition was found to significantly reduce IR-induced HSPC senescence, suggesting a role of JNK as another potential P2Y_{14} downstream modulator in IR-induced HSPC senescence. While p38 MAPK is known to play significant roles in regulating stress-induced HSPC senescence (43), the role of the JNK in SIPS is controversial (44, 45). The mechanism that enables JNK to mediate SIPS in HSPC is beyond the scope of this study. The net effects of JNK on SIPS may vary depend on the individual cellular context, intensity, and duration of JNK.

Purinergic receptors serve as sensitive sensors for cell/tissue damage and have been proposed to function as early alarm signals (9, 46, 47). Interestingly, while most of the purinergic receptors in KO mice display no aberrant phenotype under homeostatic conditions (48), the phenotypic consequences of their deficiencies become evident when KO mice are subjected to ischemia (49), infection (50), or bleeding (51, 52), suggesting the pivotal roles of purinergic receptor in a variety of pathophysiological conditions.

UDP-glucose (UDP-Glc) has been proposed as a ligand for the P2Y_{14} receptor (53). Since extracellular UDP-Glc is at high levels following mechanical stress of cells (54) or with cultured tumor cells (55), it is possible that high levels of extracellular UDP-Glc may accompany local tissue stress, triggering P2Y_{14} downstream cellular responses. Since cellular response to external UDP-Glc could vary depending on dose and time of administration, more detailed pharmacokinetic and metabolic studies of UDP-Glc would be required to determine whether UDP-Glc is a functionally relevant endogenous ligand in the context of IR-induced senescence. It should also be noted that controversy exists as to whether UDP-Glc is a functionally relevant ligand for the P2Y_{14} receptor (56, 57) raising the possibility of an as-yet-unknown P2Y_{14} ligand or ligands. Furthermore, a recent study showed that UDP-Glc can function through P2Y_{14} receptor–dependent and –independent mechanisms in the gastrointestinal (GI) tract (15). Multiple ligands are common to most of G protein coupled receptors (GPCRs), and the existence of physiologically distinct ligands for P2Y_{14} remains a possibility.

While receptor activation inhibiting apoptosis is a well-known phenomenon, the concept of a receptor whose activation inhibits its senescence has little precedent in mammals, to our knowledge, and is of interest.

Based on the ability of stem cells to repair or rejuvenate age-related tissue damage, it is conceivable that stem cell aging contributes to tissue aging. However, the issue of whether or not stem cell aging correlates with organismal aging is still the subject of debate, since the pool size and regenerative capacity of tissue-specific stem cells do not necessarily predict the life span of an organism (58). The present study shows how P2Y_{14} deficiency affected radiation-induced senescence in a subset of HSPC cells. Therefore, without further data, it is premature to predict whether P2Y_{14} has an impact on tissue or organismal aging. Whether receptor alteration of cellular stress tolerance can have other physiologic effects is yet to be explored in our mammalian model. However, it is of interest that the invertebrate gene Methuselah is also a GPCR that modulates cellular response to varying types of stress and is a known regulator of life span in Drosophila melanogaster (59). Whether P2Y_{14} may serve as a mammalian parallel of a receptor signaling in response to extracellular conditions, thereby modulating the impact of stress on organismal function and aging, is an intriguing issue for future study.

Methods

Animals and treatment. P2Y_{14} mice (15) were kindly provided under material transfer agreement (MTA) by GlaxoSmithKlein (Platform Technology and Science). Briefly, the mouse P2y14 gene (892 bp of coding sequence) was deleted by replacement with the IRES-lacZ-polyA-neo cassette, resulting in the removal of the last 6 predicted TM domains out of the 7-transmembrane domain of P2Y_{14} protein. Homozygous KO mice were obtained by mating HT. Unless otherwise stated, all experiments were performed with littermates as control. The genotyping was performed by PCR analysis as
previously described (15). NAC (100 mg/kg) was injected s.c. p38 MAPK inhibitor (SB202190, 2 mg/kg) and JNK inhibitor (SP-600125, 10 mg/kg) were administered i.p. 30 minutes before and immediately after TBI. P2Y14 WT and KO mice were injected i.v. with a single dose of PTX (0.8–1 mg/mouse) 30 minutes before TBI (6 Gy). BM cells were isolated immediately after TBI and transplanted into recipient animals (CD45.1.2).

CFU assays. BM cells were seeded at a concentration of 1.5 × 10⁴/35 mm dish for CFU-GEMM assay (Methocult 3434, Stem Cell Tech.). All assays were performed in triplicate. The colonies were counted on day 12.

Competitive long-term repopulating cell assay. Competitive repopulation assay was performed using CD45.1.2 mice as recipients, which were generated by mating SJL/J and CD45.1 female with B6 (cd45.1) male mice. BM cells from P2Y14−/− (B6, CD45.1) and then transplanted into lethally irradiated (10–11 Gy) recipient mice (CD45.1.2). P2Y14−/− BM contains a higher frequency of HSPCs than WT BM (Supplemental Figure 3). When P2Y14−/− BM cells were mixed at 1:1.2–1.3 ratio with congenic WT BM cells, equal contributions from the WT versus P2Y14−/− cells were seen in PB of recipient mice over a 1-year post-transplant period. PB and/or BM cells were collected from recipient mice at various stages of posttransplant periods and treatments, and the ratio of CD45.1+/CD45.2 was assessed by flow cytometry (Beckman Coulter).

Antibodies and flow cytometry. The cells were stained with lineage antibody cocktails and c-Ki and Sca-1 antibodies. The following lineage antibodies were purchased from eBioscience: PE-Cy7–conjugated anti-CD3 (CT-CD3), anti-CD4 (CT-CD4), anti-CD8 (CT-CD8), anti-CD45R (RA3-6B2), anti-CD11b (M1/70.15), anti–Gr-1 (RB6-8C5), and anti–TER-119 (TER-119). Sca-1 and c-Ki antibodies were from BD Biosciences—Pharmingen. FITC or PE-Texas red–conjugated anti-CD34 (RAM34) antibodies were from eBioscience. PerCP-cy5.5–conjugated anti-CD150 (TC15-12F1.2) and Pacific blue–conjugated anti-CD48 (HM48-1) antibodies were from Biolegend. FITC-conjugated anti-p16INK4a was purchased from Santa Cruz Biotechnology Inc. Pacific blue–conjugated anti-p38 MAPK was from Cell Signaling. LSK cells were sorted by MoFlo (DakoCytomation). P2Y14−/− antibodies (Alomone Labs) was biotinylated with EZ-Link Micro-PEO-Biotinylation Kit (Thermo Scientific). PE-conjugated streptavidin antibody (eBioscience) was used as a secondary antibody.

Q-RT-PCR. Cells were stained with fluorescently labeled antibodies, as indicated, and directly sorted into PCR tubes containing lysis buffer. RNA was isolated using RNeasy Micro kit (Qiagen) from sorted cells. Reverse transcription of RNA was performed using QuantiTech Reverse Transcription Kit (Qiagen). All primers for Q-PCR were purchased from Applied Biosystems.

Immunoblotting. MFIs were collected at each passage. Cell lysates were subjected to SDS-PAGE analysis. Primary antibodies for p16INK4a, p21Cip1, pRB, and pRb were purchased from Santa Cruz Biotechnology Inc.

ROS, apoptosis, JC-1 analysis. Five to eight hours after IR treatment, BM cells were stained with lineage antibody cocktails and c-Ki and Sca-1 antibodies. For the measurement of mitochondrial ROS, MitoSOX Red (Molecular Probes) was applied to LSK cells (5 μM final concentration) that were stained with APC-conjugated anti–c-Ki, FITC-conjugated anti-Sca-1, and PE/Cy7-conjugated anti-lineage markers. JC-1 ratio was evaluated by FL2/FL1 (red/green) using flow cytometry.

SA-β-gal staining. MEF cells were incubated with SA-β-gal solution (1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, and 2 mM MgCl₂ in PBS, pH 6.0) overnight at 37°C and then observed under a light microscope. For embryo staining, embryos (E18.5) were fixed in 4% paraformaldehyde for 30 minutes on ice and stained as described above. Donor-derived (CD45.2) LSK and CD150+CD48 LSK cells were analyzed for SA-β-gal activity using a fluorescent β-gal substrate, C₁₄FDG (Molecular Probes), as previously described (60, 61). Briefly, cells were stained for LSK and CD150+CD48 LSK cells as indicated above, followed by C₁₄FDG staining at a final concentration of 33 μM.

Cell-cycle analysis. BM cells were stained for LSK cells and fixed with Cytofix/Cytoperm buffer (BD Biosciences—Pharmingen). After washing once with Perm/Wash buffer (BD Biosciences—Pharmingen), cells were permeabilized with Cytoperm Plus buffer (BD Biosciences—Pharmingen). Cells were then incubated with Ki-67 antibody (BD Biosciences—Pharmingen) for 30 minutes at room temperature, which was followed by DAPI (Invitrogen) staining.

Serial transplantation. All recipient animals were conditioned as described above. At 2 months after transplantation, BM cells from primary recipients were pooled and transplanted into secondary recipient animals.

Statistics. Data are expressed as mean ± SD. and P values were determined by 1-tailed or 2-tailed Student’s t test as indicated in the figure legends. P < 0.05 was considered significant.

Study approval. All studies were approved by GlaxoSmithKlein, the Harvard Stem Cell Institute, and the University of Pittsburgh’s IACUC and were in accordance with GlaxoSmithKlein, Harvard University, and the University of Pittsburgh’s policies on the care, welfare, and treatment of laboratory animals.

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Address correspondence to: Byeog Chel Lee, University of Pittsburgh Cancer Institute, Hillman Cancer Center, 5117 Centre Avenue, Pittsburgh, Pennsylvania 15213, USA. Phone: 412.623.2285; Fax: 412.623.7828; E-mail: lee65@upmc.edu. Or to: David Scadden, Massachusetts General Hospital, Center for Regenerative Medicine, 185 Cambridge Street, CPZN-4265A, Boston, Massachusetts 02114, USA. Phone: 617.726.5615; Fax: 617.724.2662; E-mail: david_scadden@harvard.edu.

The purinergic P2Y14 receptor axis


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