Response

Homing defect in hematopoietic cells from Fanconi anemia patients

We thank O’Neill and colleagues for their comments on our paper.1 We agree that assays of clonogenicity and hematopoietic repopulation are not sufficient for defining homing. However, we argue that CFC (colony-forming cell) homing assay is an acceptable in vivo approach to assess the homing capacity of human hematopoietic progenitor cells in a mouse bone marrow (BM) transplantation model.2,3 We also share the concern of the authors of the letter that results with whole BM cells may not reflect stem and progenitor cell behavior. We must point out that with current technology, it is impossible to conduct the described experiments using purified hematopoietic stem/progenitor (HSC/P) cells from Fanconi anemia (FA) patients, simply because such immature stem cells are rare in FA patients. Indeed, previous studies from our institute with 54 FA patients evaluated in the Fanconi Anemia Comprehensive Care Center at Cincinnati Children’s Hospital Medical Center show that FA patients had a 2-fold reduction in BMcellularity and a 6-fold reduction in CD34+ cell content compared with healthy adults.4 Given the inability to collect quantities of HSC/P cells sufficient to conduct the experiments described in our paper, we have instead used BM mononuclear cells as the initial input of progenitor cells from FA BM. We have determined nonobese diabetic/severe combined immunodeficient (NOD/SCID) BM homing of human progenitors by 2 different assays: progenitor clonogenic (CFC homing) assay and human CD45/CD34 flow cytometric analysis of human progenitors homed in the BM of NOD/SCID recipients. Although these 2 methodologies are very different, they serve to be mutually confirmative, thus validating the reliability of the assays and accuracy of the data.

We are pleased to learn that the authors of the letter have confirmed our adhesion results in their Fancc−/− mouse model. It is true that some of the results were obtained from Epstein-Barr virus (EBV)-transformed lymphoblasts. However, it should be noted that our control lymphoblasts from normal donors are also EBV transformed. In addition, we showed decreased cdc42 activity in lymphoblasts derived from FA-C and FA-G patients (see Figure S1 in Zhang et al). Thus, we do not agree with the authors of the letter that the difference in cdc42 activity is due to differential responses by FA cells to EBV immortalization.

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References


To the editor:

Nuclear factor-κB is not essential for NADPH oxidase activity in neutrophils from anhidrotic ectodermal dysplasia patients

Patients suffering from anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) fail to activate the canonical nuclear factor-κB (NF-κB) pathway due to mutations in the IKK (inhibitory κB kinase gamma) gene encoding IKKγ, also known as NEMO (NF-κB essential modulator).1 This results in a combined cellular and humoral immune defect. In a recent study by Luengo-Blanco et al, the authors conclude that NF-κB activity is required for the transcription of the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase genes CYBB and NCF1, encoding gp91phox and p47phox, respectively.2 Both proteins are essential components of the NADPH-oxidase complex, and in patients with chronic granulomatous disease (CGD) failure to express these proteins results in a severe immunodeficiency.3 Luengo-Blanco et al base their conclusions on studies with Epstein-Barr virus (EBV)-transformed B-cell lines from patients evaluated with EDA-ID as well as studies with pharmacologic inhibitors of NF-κB activation in the monocytic cell line U937 and repressor transfected U937 cells. In these model systems, expression of gp91phox and p47phox was severely impaired, resulting in a failure to activate the NADPH oxidase upon stimulation with phorbol 12-myristate 13-acetate (PMA), similar to EBV-transformed B cell lines from CGD patients.2

Recently, we had the opportunity to perform functional tests with primary neutrophils from 3 different EDA-ID patients. The patients were 1, 2 and 5 years of age at the time of investigation. The second patient was the brother of the third. All 3 patients had variable degrees of dermatitis and minor peripheral lymphadenopathy since birth, as well as recurrent bacterial infections, and had the pale, sparse hair and conical incisors typical for EDA-ID patients.1 The genetic defects were all identified in the IKKγ gene resulting in a stop codon (p.Q365X) in exon 9 (c.1093C>T) in one patient and a missense mutation (p.Q205P) in exon 5 (c.614A>C) in the other 2 patients. NF-κB activation was severely impaired in all 3 patients, as demonstrated in Figure 1A by a significantly reduced production of interleukin-8 (IL-8), when determined by ELISA after overnight stimulation of the isolated neutrophils with lipopolysaccharide (LPS,
Figure 1. Neutrophils from EDA-ID patients display normal NADPH-oxidase activity. Neutrophils were isolated from heparinized peripheral blood of patients and controls over isonicotinic Percoll, as described. (A) Cells were cultured overnight in HEPES medium (132 mM NaCl, 20 mM HEPES, 6 mM KCl, 1 mM MgSO4, 1.2 mM К2HPO4, 1 mM CaCl2, 5 mM glucose and 2% (vol/vol) human serum albumin, pH 7.4) in the presence of 10 ng/mL LPS and 50 ng/mL LBP at a concentration of 5 × 106/mL, after which the IL-8 concentration was determined in the supernatant by ELISA, according to the manufacturer’s instructions (Sanquin Reagents, Amsterdam, The Netherlands). Data represent the means (± SEM) of 3 independent experiments performed in duplicate. Statistical significance was determined by a Student *t* test. *P* = .0173. (B) NADPH-oxidase activity was assessed as hydrogen peroxide production determined by an Amplex Red kit (Molecular Probes). Neutrophils (106/mL) were stimulated with 1 μg/mL bare or serum-treated zymosan (STZ), 100 ng/mL phorbol myristate acetate (PMA) or PAF + FMLP (formyl-methionyl-leucylphenylalanine; both 1 μM, added simultaneously), in the presence of 0.5 μM Amplex Red and 1 U/mL horseradish peroxidase, as described. Fluorescence was measured at 30-second intervals for 20 minutes on a Spectra Fluor Plus spectrophotometer (Tecan, Zürich, Switzerland). Maximal slope of H2O2 release was assessed over a 2-minute interval. Data represent the means (± SEM) of 3 independent experiments performed in duplicate. No significant difference was found between patient and control cells. (C) Expression of gp91phox and p67phox was assessed on Western blot. Freshly isolated neutrophils of a healthy control donor (C) or an EDA-ID patient (P) were stimulated for 2 hours with 10 ng/mL TNF-α (+) or left unstimulated (−), as indicated. Afterward, cell lysates were prepared and analyzed on Western blot for the indicated targets. Cells (1.5 × 106) were loaded in each lane and, in addition, ASC expression is shown as a loading control. Mouse anti-gp91-phox (clone 48) was obtained from Sanquin Reagents, rabbit anti-p67-phox was obtained from Upstate Biotechnology (Lake Placid, NY), mouse anti-ASC was obtained from MBL International (Woburn, MA). IRDye680 or 800CW conjugated secondary antibodies were obtained from LI-COR Biosciences (Lincoln, NE). The blots were analyzed with an Odyssey Infrared Imager (LI-COR).

10 ng/mL) and lipid binding protein (LBP, 50 ng/mL). However, in contrast to the results from Luengo-Blanco et al, NADPH-oxidase activity was completely normal in the neutrophils of these EDA-ID patients upon stimulation with various stimuli, as shown in Figure 1B. In addition, expression of both gp91phox and p67phox were found to be relatively normal in the neutrophils of the patients (Figure 1C). This suggests that, at least in primary neutrophils, NEMO-dependent NF-κB activation is not required for the expression and function of the NADPH oxidase. Instead, the recurrent bacterial infections in our EDA-ID patients seem to be due to the poor antibody reactivity against (encapsulated) pathogens (not shown), possibly combined with a failure to produce proinflammatory cytokines to recruit phagocytes to the site of infection (Figure 1A).

We suggest that the results of Luengo-Blanco et al, which were all obtained from in vitro–cultured model cell lines, can be explained by a failure to differentiate these NF-κB–deficient model cell lines in vitro to fully functional phagocytes or B cells. Primary phagocytes, or at least neutrophils, that differentiate in vivo under different circumstances, do not appear to depend on NF-κB activity for the expression of the NADPH oxidase.

**References**

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Bram J. van Raam, Robin van Bruggen, Anton T. J. Tool, Machiel H. Jansen, Adilia Warris, Stephen Jolles and Taco W. Kuijpers