J. Ky. Acad. Sci. 70(2):145–151. 2009.

Analysis of Modulation of Foxp3 Expression in $CD4^+CD25^+$ Regulatory Cells from NOD Mice

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ABSTRACT

$CD4^+CD25^+$ regulatory cells control the development of autoimmunity, including type 1 diabetes, and the transcription factor Foxp3 is crucial for the development and function of these cells. The decreased effectiveness of regulatory T cell function in diabetes-prone NOD mice is due to the failure of NOD APC to activate regulatory T cells properly. In the present study, we examined the parameters that modulate Foxp3 expression in $CD4^+CD25^+$ regulatory cells. We found that $CD4^+CD25^+$ cells from congenic diabetes-resistant NOD mice exhibited intermediate levels of Foxp3 compared with regulatory cells from B6 mice and Sick NOD mice. Using an *in vitro* Foxp3 induction system, we found that APC from diabetes-resistant congenic NOD mice also exhibited an intermediate ability to maintain expression of Foxp3 in $CD4^+CD25^+$ cells. Finally, NOD APC pre-treated with a microbial component, lipoteichoic acid, regained their ability to maintain Foxp3 expression in $CD4^+CD25^+$ cells *in vitro*. APC from CFA-treated NOD mice that were transferred into recipient NOD mice could induce Foxp3 expression in $CD4^+CD25^+$ cells *in vivo*. Altogether, these data suggest that Foxp3 expression in $CD4^+CD25^+$ cells from NOD mice can be modulated by optimal stimulation by APC.

KEY WORDS: Type 1 diabetes, regulatory cells, Foxp3 expression, antigen presenting cells, *Idd*

INTRODUCTION

Non obese diabetic (NOD) mice develop spontaneous diabetes that resembles human Type I diabetes. The disease incidence varies from 60 to 90% in female NOD mice depending on the animal facility and is much lower in males. NOD mice appear to have a dysregulated immune response, including deficiency in two regulatory cell populations, NKT and $CD4^+CD25^+$ regulatory T cells (Gombert 1996; Wu 2002) and antigen-presenting cell (APC) function (Serreze 1993; Piganelli 1998; Dahlen 2000; Lee 2000). $CD4^+CD25^+$ regulatory T cells play a crucial role in controlling autoimmune disease development, including type 1 diabetes. Although controversial, the percentage and function of these cells have been found to be altered in NOD mice (Salomon 2000; Kishimoto 2001; Wu 2002; Alard 2006) and diabetic patients (Kukreja 2002). We recently have shown that the defect in regulation observed in NOD mice appears to lie in the inability of NOD APC to activate $CD4^+CD25^+$ regulatory T cells (Alard 2006). Moreover, we have compelling data showing that complete Freund's adjuvant (CFA) injection into NOD mice restores functional APC and regulatory cells and prevents diabetes development (Manirarora 2008).

$CD4^+CD25^+$ regulatory T cells account for 5–10% of $CD4^+$ cells in healthy mice and humans and are crucial for controlling the development of autoimmune diseases. Although the mechanisms of action of $CD4^+CD25^+$ regulatory T cells are still controversial (Sakaguchi 2004), these cells absolutely require activation (Takahashi 1998; Thornton 1998), presumably by APC, to mediate regulation. Recently, a transcription factor, Foxp3, has been found to be critical for $CD4^+CD25^+$ regulatory T cell development and function. $CD4^+CD25^+$ regulatory T cells from Foxp3-deficient mice are not functional, and naive T cells forced to express Foxp3 exhibit regulatory function (Fontenot 2003; Hori 2003; Khattri 2003). Few studies have confirmed the relationship between Foxp3 and regulatory cell function using transgenic mice expressing GFP under the control of the Foxp3 promoter (Sakaguchi 2004; Fontenot 2005). More importantly, a recent study has found a correlation between levels of Foxp3 expression and regulatory function (Wan 2007) suggesting that failure to maintain optimal Foxp3 expression could compromise regulatory cell function and lead to autoimmune disease development.

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$CD4^+CD25^+$ regulatory cells from NOD mice were found to express diminished level of Foxp3 at the mRNA level (Pop 2005). We have confirmed this observation at the protein level and have shown that NOD APC were less efficient at maintaining Foxp3 expression in $CD4^+CD25^+$ regulatory T cells from NOD mice (Manirarora 2008). In the current study, we have investigated in more detail the defect in Foxp3 expression in $CD4^+CD25^+$ regulatory T cells from NOD mice. We have examined whether the levels of Foxp3 expressed by $CD4^+CD25^+$ regulatory T cells was normal in diabetes-resistant congenic NOD mice. We also have examined whether APC from diabetes-resistant congenic NOD mice were able to maintain Foxp3 expression in $CD4^+CD25^+$ regulatory T cells from NOD mice. Finally we assessed whether pre-activation of NOD APC with microbial components could restore normal levels of Foxp3 in NOD $CD4^+CD25^+$ regulatory T cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

Mice

Eight-fifteen week-old C57BL/6 and NOD female mice (Jackson Laboratory, Bar Harbor, ME) and congenic *Idd*3/5 and *Idd*9 NOD female mice (Taconic, Hudson, NY) were maintained under specific pathogen-free conditions as described in the Institutional Animal Care and Use Committee guidelines.

Antibodies and Flow Cytometry

APC-anti-CD25, PerCP-anti-CD4 antibodies were purchased (BD Pharmingen, San Diego, CA). One million cells were incubated with Fc block and labeled with antibodies for 20 min in DPBS 1% FCS, 0.1% $NaNO_3$, and washed twice. For Foxp3, cells were intracellularly labeled with PE-anti-Foxp3 antibodies (eBioscience, San Diego, CA) according to the manufacturer's instructions. Cells were analyzed by FACS® using a FACScalibur (Becton Dickinson, Palo Alto, CA).

T cell Depletion of Spleen Cells

Spleen cells were incubated in lysis buffer (RPMI 1640 with 1M Hepes and 0.3 g BSA) containing anti-mouse CD90 antibody (Cedarlane, Hornby, Ontario, Canada), then with Low-Tox-M rabbit complement (1:10; Cedarlane, Hornby, Ontario, Canada). The purity of the $CD3^-$ cells was consistently >95%. In some case, $CD3^-$ cells were stimulated overnight in the presence of 10 μg/ml of lipoteichoic acid (LTA),

Cell Culture for Evaluation of Foxp3 Maintenance

$CD4^+CD25^+$ regulatory T cells from three mice were pooled and sorted to >95% purity (MoFlo®, DakoCytomation, Fort Collins, CO) and cultured in multiple wells ($2–4 \times 10^4$ cells/well) in complete media (RPMI 1640, 10% heat-inactivated FCS, 2 mM glutamine, 10 mM HEPES, 100 U/ml pen G sodium, 100 μg/ml strep sulfate, and 1×10^{-5} 2 ME) for 18 hrs with irradiated T cell-depleted spleen cells (APC; 1×10^5) pooled from three mice and anti-CD3 antibody (0.5 μg/ml).

Local Adoptive Transfer to Evaluate Foxp3 Maintenance *in vivo*

Complete Freund's adjuvant (CFA) was prepared using non-viable desiccated *Mycobacterium tuberculosis* (H37 RA, Difco Laboratories, Detroit, MI) at 1 mg/ml in PBS and emulsified in incomplete Freund's Adjuvant (IFA; Sigma-Aldrich, St Louis, MO). Eight week-old NOD mice were injected subcutaneously with 100 μl of PBS or complete Freund's adjuvant (CFA). Three weeks later, spleen cells were harvested, depleted of T cells, and 1×10^6 cells injected subcutaneously in the footpad of 6–8 week-old NOD mice. After four days, popliteal LN were collected, and cells were labeled for Foxp3.

Statistical Analysis

Data were analyzed using the student's *t*-test. Each experiment was repeated with reproducible results at least 2 times. One representative experiment is shown in each figure or table.

RESULTS

Comparison of Foxp3 Expression in $CD4^+CD25^+$ Regulatory Cells from Diabetes-Resistant Congenic NOD Mice and Diabetes-Prone NOD Mice

Multiple genetic loci control disease susceptibility in NOD mice, and one of these loci include the MHC class II I-$A\beta^{g7}$ allele. Furthermore, congenic NOD mice bearing

Table 1. $CD4^+CD25^+$ regulatory cells from congenic diabetes-resistant mice exhibit intermediate levels of Foxp3 by comparison with cells from B6 and Sick NOD mice.

$CD4^+CD25^+$ cells	% $Foxp3^{+a}$	Foxp3 MFI[a]
B6	88	310
$B6^{g7}$	93	230
*Idd*3/5	94	220
*Idd*9	93	223
Sick NOD	87	175

[a] Splenic cells were analyzed for Foxp3 expression by FACS® after gating on $CD4^+CD25^+$ cell and percent of $Foxp3^+$ cells and mean fluorescent intensity (MFI) for Foxp3 are represented. Representative results from one of two experiments are shown.

one or more loci termed insulin-dependent diabetes (*Idd*) loci that are linked to diabetes resistance or susceptibility, such as *Idd*3 (Lyons 2000), *Idd*5 (Kissler 2006) or *Idd*9 (Lyons 2000) are highly protected from the occurrence of type-1 diabetes (Maier 2005; Hamilton-Williams 2007). Because we previously had found that $CD4^+CD25^+$ regulatory cells from NOD mice exhibited a lower Foxp3 expression than cells from B6 mice (Manirarora 2008), we first examined whether $CD4^+CD25^+$ regulatory cells from diabetic-resistant *Idd*3/5 and *Idd*9 congenic NOD mice exhibited normal levels of Foxp3. Cells from spleens of four mice were pooled and labeled with anti-CD25, anti-CD4 and anti-Foxp3 antibodies and analyzed by FACS. We found that $CD4^+CD25^+$ regulatory cells from congenic diabetes-resistant mice exhibit intermediate levels of Foxp3 compared with cells from B6 (normal control) and NOD mice (Table 1) Furthermore, $CD4^+CD25^+$ regulatory cells from $B6^{g7}$ mice that express the MHC class II I-$A\beta^{g7}$ allele of NOD mice also exhibited intermediate Foxp3 expression, suggesting that the allele I-$A\beta^{g7}$ may be involved in the intermediate Foxp3 expression observed in $CD4^+CD25^+$ regulatory cells from congenic diabetes-resistant mice.

Comparison of the Ability of APC from Diabetes-Resistant Congenic NOD Mice and Diabetes-Prone NOD Mice to Maintain Foxp3 Expression *in vitro*

We had shown in previous studies that APC from NOD mice failed to optimally activate $CD4^+CD25^+$ regulatory cells *in vitro*, as shown by decreased suppression of effector cell proliferation (Alard 2006) and lower maintenance of Foxp3 expression *in vitro* (Manirarora 2008). In the current study, we first tested whether $CD4^+CD25^+$ regulatory cells from eight week-old pre-diabetic or 12–15 week-old Sick NOD mice responded differently to APC stimulation for the maintenance of Foxp3 expression using an *in vitro* assay that we have developed (Manirarora 2008). Indeed, $CD4^+CD25^+$ regulatory cells from Sick NOD mice exhibited lower levels of Foxp3 after stimulation *in vitro* with B6, pre-diabetic or Sick NOD APC (Table 2, rows 1–3) compared with $CD4^+CD25^+$ regulatory cells from pre-diabetic NOD mice (Table 2, rows 4–6). Similarly, APC from pre-diabetic NOD mice were more efficient than APC from Sick NOD mice at maintaining Foxp3 expression in $CD4^+CD25^+$ regulatory cells from pre-diabetic (Table 2, rows 5&6) or Sick (Table 2, rows 2&3) NOD mice.

We next tested whether APC from diabetes-resistant mice could sustain normal levels of Foxp3 *in vitro*. Spleens were harvested from age-matched B6, $B6^{g7}$, *Idd*3/5, *Idd*9 and sick NOD mice. Cells were pooled from each group and depleted of T cells and cultured overnight with sorted $CD4^+CD25^+$ regulatory cells in the presence of anti-CD3 antibody. At the end of the culture, cells were labeled with anti-CD25, anti-CD4 and anti-Foxp3 antibodies and analyzed by flow cytometry. We found that APC from congenic diabetes-resistant mice had an intermediate ability to sustain Foxp3 expression *in vitro* compared with APC from either B6 or sick NOD mice (Table 3). Furthermore, APC from $B6^{g7}$ mice also were less efficient at maintaining Foxp3 expression *in vitro*. The intermediate expression of Foxp3 found in these strains of mice (Table 1) appeared to correlate with the intermediate ability of APC from these mice to maintain Foxp3 expression in $CD4^+CD25^+$ regulatory cells.

CEffects of *in vitro* and *in vivo* Activation of APC on Induction of Foxp3 Expression in $CD4^+CD25^+$ Regulatory Cells

Multiple studies have reported that Complete Freund's adjuvant (CFA), which contains *Mycobacterium tuberculosis*, can prevent diabetes development when injected into young NOD mice (McInerney 1991; Qin

Table 2. Comparison of APC and $CD4^+CD25^+$ regulatory cells from pre-diabetic NOD and Sick NOD mice for the maintenance of Foxp3 expression *in vitro* upon overnight culture of $CD4^+CD25^+$ T cells with APC and anti-CD3 antibody.

APC[a]	$CD4^+CD25^+$ cells	% $Foxp3^{+b}$	Foxp3 MFI[b]
B6	Sick NOD	90	259
Pre-diabetic NOD	Sick NOD	98	181
Sick NOD	Sick NOD	94	146
B6	Pre-diabetic NOD	94	281
Pre-diabetic NOD	Pre-diabetic NOD	98	227
Sick NOD	Pre-diabetic NOD	88	171

[a] Irradiated T-cell depleted spleen cells.

[b] Cells pooled from multiple wells were analyzed for Foxp3 expression by FACS® after gating on CD4+CD25+ cell and percent of Foxp3+ cells and mean fluorescent intensity (MFI) for Foxp3 are represented. Representative results from one of two experiments are shown.

1993; Lee 2004). We found previously that $CD4^+CD25^+$ regulatory cells from CFA-treated NOD mice express normal levels of Foxp3, and APC from those mice recover their ability to stimulate regulatory cell function and maintenance of Foxp3 expression *in vitro* (Manirarora 2008). In the current study, we first tested whether exposure of APC to components of microorganisms *in vitro* also could render NOD APC capable of sustaining Foxp3 expression in $CD4^+CD25^+$ regulatory cells. Because CFA mediates its effect on APC via TLR2 (Lim 2002), we used a TLR2 ligand, lipoteichoic acid (LTA), isolated from *Staphylococcus aureus*. Spleens were harvested from sick NOD or B6 mice, and cells were depleted of T-cells and either left unstimulated or stimulated overnight with LTA. On the following day, cells were irradiated, washed, and used as APC in overnight cultures with $CD4^+CD25^+$ regulatory cells purified from either B6 or sick NOD mice. Cells were then labeled with anti-CD4, anti-CD25, and anti-Foxp3 antibodies and analyzed by flow cytometry. LTA-stimulated APC from sick NOD mice sustained Foxp3 expression *in vitro* compared with unstimulated APC from sick NOD mice (Table 4} Taken together, these data suggest that APC stimulated with LTA recover their ability to stimulate $CD4^+CD25^+$ regulatory cells *in vitro*.

Table 3. APC from diabetes-resistant congenic mice sustain intermediate Foxp3 expression *in vitro* upon overnight culture of $CD4^+CD25^+$ T cells with APC and anti-CD3 antibody.

APC[a]	$CD4^+CD25^+$ cells	% $Foxp3^{+b}$	Foxp3 MFI[b]
B6	Sick NOD	94	234
$B6^{g7}$	Sick NOD	97	206
*Idd*3/5	Sick NOD	96	180
*Idd*9	Sick NOD	94	190
Sick NOD	Sick NOD	98	167

[a] Irradiated T-cell depleted spleen cells.

[b] Cells pooled from multiple wells were analyzed for Foxp3 expression by FACS® after gating on CD4+CD25+ cell and percent of Foxp3+ cells and mean fluorescent intensity (MFI) for Foxp3 are represented. Representative results from one of two experiments are shown.

We next tested whether APC collected from CFA-treated mice could enhance Foxp3 expression *in vivo*. Spleen cells were harvested from CFA-treated or PBS-treated NOD mice, depleted of T cells, and 1×10^6 cells injected in the left footpad of 8 week-old NOD mice. Four days later, the left popliteal LN were collected, and cells labeled with anti-CD25, anti-CD4, and anti-Foxp3 antibodies and analyzed by flow cytometry. Foxp3 expression was significantly enhanced in $CD4^+CD25^+$ cells harvested from the LN of mice that had received APC from CFA-treated NOD mice compared to $CD4^+CD25^+$ cells harvested from the LN of mice that had received APC from PBS-treated NOD mice (Figure 1). These data suggest that APC activated *in vivo* with CFA and injected into recipient mice were able to enhance locally expression of Foxp3 in regulatory cells.

DISCUSSION

Foxp3 appears to be a critical transcription factor for $CD4^+CD25^+$ regulatory T cell development and function (Fontenot 2003; Hori 2003; Khattri 2003). Furthermore, regulatory function correlates with levels of Foxp3 expression (Wan 2007), suggesting that failure to maintain optimal Foxp3 expression could compromise regulatory cell function and lead to autoimmune disease development. Because $CD4^+CD25^+$ regulatory cells from NOD mice were found to express diminished

Table 4. Lipoteichoic acid (LTA) restores the ability of NOD APC to induce Foxp3 expression in CD4+CD25+ cells upon overnight culture of CD4+CD25+ T cells with APC and anti-CD3 antibody.

APC[a]	CD4+CD25+ cells	% Foxp3+[b]	Foxp3 MFI[b]
B6	Sick NOD	82	177
Sick NOD	Sick NOD	86	134
LTA-B6	Sick NOD	85	212
LTA-Sick NOD	Sick NOD	87	189

[a] Irradiated T-cell depleted spleen cells.

[b] Cells pooled from multiple wells were analyzed for Foxp3 expression by FACS® after gating on CD4+CD25+ cell and percent of Foxp3+ cells and mean fluorescent intensity (MFI) for Foxp3 are represented. Representative results from one of two experiments are shown.

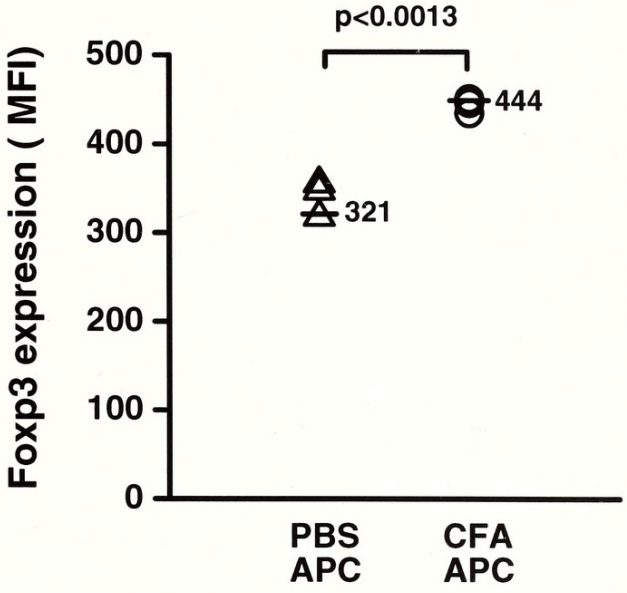

Figure 1. Induction of Foxp3 expression in CD4+CD25+ cells upon subcutaneous injection of CFA-stimulated APC. Popliteal LN cells from mice injected with PBS or CFA-stimulated APC were harvested, labeled with anti-CD4, CD25 and Foxp3 antibodies, and analyzed by FACS® after gating on CD4+CD25+ cells. Mean fluorescent intensity (MFI) for Foxp3 are represented.

levels of Foxp3 at the mRNA (Pop 2005) and protein level (Manirarora 2008), in the current manuscript we analyzed the modulation of expression of Foxp3 in CD4+CD25+ T cells from diabetes-prone NOD and diabetes-resistant congenic NOD mice. We showed that expression of Foxp3 was affected by three parameters, the expression of MHC class II I-Aβ^{g7} allele by APC, the disease state, and optimal stimulation by APC.

The expression of Foxp3 in CD4+CD25+ T cells from diabetes-resistant congenic NOD mice that express the MHC class II I-Aβ^{g7} allele was lower than that from B6 mice but higher than that of NOD mice. Similar results were found in in CD4+CD25+ T cells from B6 mice that expressed that same class II allele (B6^{g7} mice). We had previously shown that CD4+CD25+ T cells from pre-diabetic NOD mice also expressed an intermediate expression of Foxp3 (Manirarora 2008). Similarly, the ability of APC from diabetes-resistant congenic and pre-diabetic NOD mice to maintain Foxp3 expression also was intermediate suggesting that the MHC class II I-Aβ^{g7} allele is involved at some level in the decrease in Foxp3 expression in CD4+CD25+ T cells from NOD mice. The MHC class II I-Aβ^{g7} allele is one of multiple genetic loci that control disease susceptibility in NOD mice. It has been reported that coexpression of protective βchains such as I-Aβ^{d} or I-Aβ^{k} with the endogenous disease-prone βchain (I-Aβ^{g7}) in about 15% of bone marrow-derived haematopoietic stem cells provides protection against the development of insulitis and diabetes (Tian 2004). However, the MHC class II I-Aβ^{g7} allele is not sufficient to induce disease as mice expressing the defective MHC-Class II allele I-Aβ^{g7} on a C57BL/6 (B6) background (B6^{g7} mice) do not develop diabetes (Wong 2005). Furthermore, the ability of APC to maintain Foxp3 expression is lower if CD4+CD25+ regulatory cells from sick NOD mice are used in the *in vitro* assay compared with pre-diabetic NOD mice, suggesting that the disease state can affect Foxp3 expression in CD4+CD25+ regulatory cells at some level. Using transgenic mice that were rendered chronically hyperglycemic beginning shortly after birth by rat insulin promoter driven expression of calmodulin (OVE26) and subsequent β cell damage (Epstein 1989), we showed that higher glucose levels have no effect on Foxp3 expression in CD4+CD25+ regulatory cells (Manirarora 2008). However, other metabolites that are produced during diabetes progression may affect APC and/or regulatory cells, thereby leading to further decreases in Foxp3 expression.

We had shown previously that activation of APC *in vivo* by treatment with CFA appeared to significantly enhance the NOD APC's ability to activate CD4+CD25+ regulatory T

cells, as indicated by increased regulatory activity both *in vivo* and *in vitro*, and restoration of an optimal regulatory cell phenotype, i.e., increased Foxp3 expression in $CD4^+CD25^+$ T cells (Manirarora 2008). Our data suggested that the effect of CFA was, at least in part, mediated through enhancement of the ability of NOD APC to act on NOD $CD4^+CD25^+$ regulatory T cells by sustaining Foxp3 expression. In the current manuscript we demonstrated that APC stimulated by CFA *in vivo* could indeed induce induction of Foxp3 expression in NOD regulatory cells *in vivo*, by performing a local adoptive transfer of CFA-treated APC and assessing Foxp3 expression in the draining LN a few days later. Because CFA, which contains desiccated *Mycobacterium tuberculosis*, appears to mediate its effect *in vivo* through TLR2 (Lim 2002), we tested whether a TLR2 ligand could reproduce the CFA effect *in vitro*. Pre-treatment with lipoteichoic acid (LTA), a TLR2 ligand, could indeed restore the ability of NOD APC to maintain Foxp3 expression in $CD4^+CD25^+$ regulatory cells *in vitro*, indicating that cell wall components targeting TLR are capable of mediating this effect. Another cell wall component of *Mycobacterium tuberculosis*, mannose-capped lipoarabinomannan, has been shown to induce expansion of $CD4^+CD25^+Foxp3^+$ regulatory T cells by binding to receptors expressed by APC (Garg 2008). There are, therefore, several potential candidates capable of enhancing the ability of APC to preferentially induce regulatory cells.

In conclusion, we have established a link between the decreased expression of Foxp3 in $CD4^+CD25^+$ regulatory cells and the expression of MHC class II I-$A\beta^{g7}$ allele by APC in NOD mice. Moreover, disease progression appear to affect Foxp3 expression as well. More importantly, the ability of NOD APC to induce/maintain Foxp3 expression in $CD4^+CD25^+$ T cells can be restored by stimulation with TLR ligand, and manipulating the ability of APC to activate/induce $CD4^+CD25^+$ regulatory T cells is a potential strategy that could be used to prevent disease.

ACKNOWLEDGEMENTS

This study was supported by a grant from the University of Louisville School of Medicine. Jean N. Manirarora was supported by a Ruth L. Kirschstein NRSA.

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