ISG15 is counteracted by Vaccinia Virus E3 protein and controls the proinflammatory response against viral infection

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Conjugation of ISG15 inhibits replication of several viruses. Here, using an expression system for assaying human and mouse ISGylations, we demonstrate that Vaccinia virus E3 protein binds and antagonizes human and mouse ISG15 modification. To study ISGylation importance in Poxvirus infection, we used a mouse model that expresses de-conjugating proteases. Our results indicate that ISGylation restricts VVΔE3L *in vitro* replication but unconjugated ISG15 is crucial to counteract the inflammatory response produced after VVΔE3L infection.
Type I interferon (IFN-α/β) is essential for controlling the replication of viruses in their mammalian hosts. Binding of IFN-α/β to its receptor activates the Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway, and consequently leads to the up-regulation of hundreds of IFN-stimulated genes (ISG) (8). One of the most highly induced genes is ISG15, which encodes a small ubiquitin-like (UBL) protein of 17 kDa that forms covalent conjugates with cellular and viral proteins mediating considerable antiviral response (1, 2). ISG15 is composed of two domains, each of which carries high sequence and structural similarity to UB (33 and 32 % for the N- and C-terminal domains, respectively (21)). Conjugation of ISG15 to its protein substrates follows similar principles as UB, requiring an E1-activating enzyme (UBE1L), an E2-conjugating enzyme (UbcH8) and an E3 ligase, which in humans is mainly Herc5 while in mice mHerc6 performs this function (6, 37).

The mechanism responsible for the antiviral activity of ISG15 is not clearly understood. ISG15 has been reported to be conjugated to proteins involved in direct or indirect antiviral activity including RIG-I, JAK1, STAT1, interferon regulatory factor 3 (IRF3) and protein kinase R (PKR) (23, 38). It has also been shown that IRF3 ISGylation prevents its ubiquitination and degradation, enhancing its translocation to the nucleus. Consequently, the relative amount of IRF3 is reduced in ISG15-/- compared to ISG15+/+ cells (22). ISG15 negatively regulates important cell signalling pathways such as RIG-I like receptor (RLR) signalling and activation of NF-κB (15, 25). On the other hand, protein deISGylation negatively regulates the JAK/STAT pathway (23). It has also been suggested that ISG15 conjugation to viral proteins have detrimental effects in viral replication. Finally, free, unconjugated ISG15 appear to have antiviral properties at least for some viruses (32).
Studies with several viruses have verified that ISG15 plays an essential role in the antiviral response (18, 28). Thus, evidence supports the antiviral activity of both conjugated and unconjugated ISG15. For Sindbis virus, the increased lethality seen in ISG15-/- mice can be rescued by a recombinant virus expressing wild-type ISG15 but not mutant ISG15, the latter not being able to form conjugates in vitro (19). In contrast, two reports showed that free ISG15, in the absence of its conjugation cascade, inhibited the release of Ebola virus-like particles (24, 26).

Free ISG15, but not ISGylation, promotes antiviral responses against Chikungunya virus infection (32). Previously, we described ISG15 upregulation using cDNA microarrays after infection of HeLa cells with the attenuated vaccinia virus (VACV) strains MVA and NYVAC, an effect not observed after infection with the virulent strain WR (12-14). Also, we have demonstrated the importance of ISG15 in the context of Poxvirus infection (11). We evaluated disease progression in ISG15-/- and ISG15+/+ mice after infection with WR and with the attenuated mutant VVΔE3L that lacks the viral early protein E3, using different routes of inoculation. We determined that E3 blocked the antiviral effect of ISG15. However, the mechanism by which E3 is able to block ISG15 is still unknown. The E3 protein also represses the host cell antiviral response by multiple mechanisms, including PKR and RNase L inhibition, two enzymes induced by IFN. When activated, PKR and RNase L trigger a global inhibition of protein synthesis and of virus replication (27, 33) through the phosphorylation of eIF-2α (for PKR) and breakdown of RNA (for RNase L). E3 also blocks induction of genes such as IFN α/β through inhibition of phosphorylation of the transcription factors IRF3 and IRF7 (29, 34) and prevention of NF-κB activation (7).

Taking into account the important role of ISG15 in establishing the antiviral state of the infected cell, several viruses have developed strategies to counteract its antiviral action. Here, we wanted...
to investigate if E3 was able to inhibit ISG15 as previously described for the NS1 protein of influenza B virus (30, 31). Influenza B NS1, a protein with structural and functional similarities to E3 (10), binds and inhibits human but not mouse ISG15 (30, 31). Our first approach was to test whether E3 protein was binding to ISG15 protein from human or mouse origin. Previously, we had described that in the mouse, ISG15 binds the E3 protein in a PKR-independent manner (11). To confirm these results and to extend these studies to the human model, pull-down experiments were performed. For these assays, expression plasmids with glutathione S-transferase (GST) fused in frame to human or mouse ISG15 were generated and coexpressed in 293T cells with plasmids encoding the entire viral E3 gene or B/NS1 that was used as control. Protein lysates were subjected to GST pull-down and precipitated proteins were analyzed by Western blot using E3, NS1 and GST-specific antibodies (Figure 1 left panels). In the whole-cell lysates (Figure 1A, lower left panels) similar expression levels of E3 and GST fusions were observed. In both cases, GST-hISG15 and GST-mISG15 precipitated with E3 (Figure 1A, upper left panels), indicating that E3 is able to interact with human and also murine ISG15. E3 has two domains: an N-terminal involved in the direct inhibition of the IFN-induced dsRNA-dependent protein kinase PKR in nuclear localization, and in Z-DNA binding (5, 16, 17); and the C-terminal domain that contains the dsRNA-binding domain required for IFN resistance and for the broad host range phenotype of the virus (3, 5). To study whether the specific region responsible of the binding between the E3 protein and ISG15 binding was the same in the murine and in the human system, we constructed plasmids expressing two portions of E3 protein: the first 165 and the last 108 amino acids. We then performed similar pull-down experiments using the two portions of the E3 protein. The C-terminus domain of E3 is needed for its interaction with the murine or the human ISG15 (Figure 1A, right panels). These results indicate that VACV
E3 has a higher binding permissibility for ISG15 from multiple species than B/NS1, because differences between mouse and human ISG15 sequence are not influencing E3 protein binding ability. However, substituting the mouse sequence (QNCSE) for the human hinge sequence (DKCDE) in human ISG15 resulted in a total loss of NS1B binding (36).

ISG15 conjugation (ISGylation) to substrate proteins occurs in a manner similar to UB conjugation, by utilizing activating, conjugating and ligating enzymes to facilitate the addition of ISG15 to specific lysine residues. Although we defined that E3 regulates ISG15 activity, the only evidence until now that suggests that E3 blocks the ISGylation process is the increased level of ISGylated complexes observed in VVΔE3L-infected mouse lung homogenates (11). To elucidate whether E3 blocks the ISGylation activity and to further gain insights on its mechanism, we performed an *in vitro* assay that allowed us to study the impact of the expression of E3 on protein ISGylation. We transfected 293 cells with ISG15 and its specific E1 (UBE1), E2 (UbcM8) and E3 (HERC5) enzymes expression plasmids to evaluate ISGylated protein levels in both, human and murine systems. Next, we compared levels of ISGylated protein after cotransfection of the E3 plasmid with the plasmids described above. Using the human ISGylation system, we observed a decrease in total ISGylation when E3 was coexpressed. This suggests that E3 might be blocking the ISGylation activity of human ISG15 (Figure 1B). When we performed similar experiments using the murine system we observed also a decrease in the ISGylation levels when the entire E3 VACV protein was cotransfected, although the efficacy of this blockage is lower than the observed in the human system (Figure 1C). Interestingly, both C- and N-terminal domains of E3 are required for ISGylation inhibition, indicating that E3 binding to ISG15 is not sufficient for ISGylation inhibition.
Host restriction of a virus is driven by its ability to counteract specific components of the innate immune response in selected species. In the context of Influenza B virus, its inability to block ISGylation in mice and in other hosts as well may contribute to limit the host range of the virus. Moreover, it might account for the increased susceptibility of ISG15−/− mice to Influenza virus infection, as previously described (20). In contrast, the capacity of VACV to counteract the ISGylation by E3 in the murine system, correlated with no differences in pathogenesis between ISG15−/− and ISG15+/+ infected with VACV as we previously reported (11).

According to these aforementioned considerations, we can speculate that subversion of ISGylation may control virus replication in mouse infection. In order to elucidate this question and to define the roles between ISG15 and ISGylation in the context VACV infection, we used a transgenic mouse model in which proteases with de-conjugating activity (OTU) are expressed (9). Therefore, although ISG15 is expressed, the ISGylation is completely repressed. We have previously reported that the attenuated VVΔE3L virus was able to grow in ISG15−/− MEFs. Our next step was to understand if this phenotype was also observed in OTU MEFs. The cytopathic effect (CPE) observed in OTU MEFs after VACV (WR strain) infection (0.1 PFU/cell, 24 h) was similar to ISG15−/− or ISG15+/+ cells (data non shown). However, the CPE after VVΔE3L infection in OTU cells was similar to the one in ISG15−/− and was markedly increased compared to the observed in ISG15+/+ cells (data non shown). VACV viral titers were slightly increased in ISG15−/− and OTU cells compared to ISG15+/+ cells (Fig. 2A). On the other hand, a significant increase of VVΔE3L yields was observed in ISG15−/− and OTU cells compared to ISG15+/+ cells (Fig.2B). In the VVΔE3L-infected ISG15−/− and OTU cells, the increase in virus titers correlated with increase in cellular mortality (Fig. 2D) indicating that VVΔE3L overcomes viral growth blockage only in the absence of ISGylation. This suggests that E3-mediated ISGylation inhibition might account for
similar levels in VACV replication in ISG15−/− ISG15+/+ or OTU cells, thus validating our in vitro assays shown in Fig.1.

To expand these studies to an in vivo model, transgenic OTU and wild type mice were inoculated intranasally with different doses of VACV (WR strain). We examined the degree of viral pathogenesis during the first days of the infection and no differences in weight loss and in mortality were detected between OTU and wild type mice (Figure 3A). Although at the higher dose of VACV, all OTU transgenic animals died while the WT animals were still alive, we do not think these are significant differences, as two days later, all the WT infected animals also succumbed to infection. This result indicates that there were no differences in the pathogenesis with VACV-infected ISG15−/− or OTU in comparison to ISG15+/+ mice. However, in the case of VVΔE3L an enhanced inflammatory response and mortality is observed only after ISG15−/− high dose infection (11). To analyze whether the cause of VVΔE3L-associated increased pathogenicity in infected ISG15−/− mice was due to a lack of ISGylation, we infected OTU transgenic mice with VVΔE3L virus. OTU, ISG15−/− and ISG15+/+ mice were inoculated intranasally with 10⁸ PFU of VVΔE3L per mouse and weight loss and mortality were evaluated. As previously described (11), VVΔE3L-infected ISG15−/− mice displayed mild disease symptoms within 3 days and 25% of them died at 1 dpi after high dose in the absence of viral replication in the lungs (Figure 3B). Surprisingly, when ISGylation was decreased due to the expression of the OTU transgene, VVΔE3L was still attenuated (Fig. 3). The animals also displayed mild signs of disease at 3 dpi but all the animals recovered at the endpoint of the experiment (Fig. 3B). Also, VVΔE3L virus could not be detected in lung lysates from neither OTU, ISG15−/− nor ISG15+/+ animals (Fig. 3C). This result suggests that the presence of un-conjugated ISG15, which is not affected in the OTU transgenic mouse, is a requirement to
maintain the attenuated phenotype of VVΔE3L virus in vivo (Figure 3A-B). Histological examination of lung tissues at 1dpi showed that ISG15+/+ or OTU animals infected with VVΔE3L virus had no inflammatory cells infiltrating the lung parenchyma. In contrast, lung sections obtained from VVΔE3L-infected ISG15-/− mice presented a severe inflammation pattern with alveolar wall thickening and infiltration of inflammatory cells (Figure 4A). Lung inflammation was also drastically reduced in infected OTU mice. These results suggest that unconjugated ISG15 is responsible to counteract the development of an exacerbated inflammatory response after a high dose of VVΔE3L virus. To analyze the state of ISGylation in the VACV- or VVΔE3L-infected mice, lungs were homogenized and ISG15 levels were determined by Western blot. While, ISG15-/− mice do not express ISG15 (Figure 4B), lungs from OTU mice showed only un-conjugated ISG15, as expected, whereas the homogenates from ISG15+/+ mice showed free ISG15 and conjugated ISG15 to cellular targets (Figure 4B). The conjugation of ISG15 to its targets proteins was considerably enhanced in lung extracts from mice infected with VVΔE3L in comparison to those infected with VACV. This result suggests that E3 also was able to inhibit the ISGylation in vivo validating our in vitro analysis (Figure 1).

Since the inflammatory response might explain the rapid signs of illness in high dose VVΔE3L-infected ISG15-/− mice, we measured serum cytokine levels (IL-6, TNF-α, IL-10, monocyte chemo-attractant protein 1, IFN-γ, and IL-12 p70) at early times post infection in the infected ISG15-/−, ISG15+/+ or OTU mice. IL-6 levels were very low and similar in serum from VVΔE3L-infected OTU or ISG15+/+ animals (Figure 4C). In contrast, ISG15-/− mice infected with VVΔE3L had 8-fold increase in serum levels of IL-6 compared to ISG15+/+ or OTU infected mice (P < 0.01; Figure 4C). There were no changes in levels of other cytokines analyzed between the groups (not shown). Cytokine and chemokine release occurs rapidly in response to
virus infection, with the aim of recruiting inflammatory leukocytes in order to limit virus replication and spread, and to induce adaptive immunity. However, prolonged expression of chemokines in the context of viral infections may be detrimental to the host provoking the called cytokine storm. Here, we demonstrated that in absence of non-conjugated ISG15, VVΔE3L infection produces an increase of IL6 that correlates with short-term morbidity and complications that include pulmonary function abnormalities indicating that free ISG15 might be involved in the down-regulation of IL-6 induction, facilitating a balanced, non-pathogenic innate immune response. To verify that the morbidity was induced by VVΔE3L infection, we evaluated the survival rate of ISG15-/- and ISG15+/+ mice inoculated with different inflammatory agents, such as polyI:C (10 mg/Kg), LPS (15 mg/Kg) and Zymosan (15 mg/Kg) (Fig 4D). In all the cases the survival kinetics was similar between both types of mice and no differences in the IL-6 levels in the serum of the treated mice were observed (Fig. 4 E). This result indicates that VVΔE3L infection was the cause of the mortality and also caused exacerbated levels of IL-6 after ISG15-/- mice. Whether these effects are due to the absence of E3 directly or another process, the mechanisms of this ISG15-dependent, ISGylation-independent regulation remain to be determined, but it appears that ISG15 controls the antiviral response by multiple ways, depending on whether it is or nots conjugated. Also, it is important to underscore that free ISG15 becomes secreted and the lack of secreted ISG15 is associated with severe mycobacterial disease in both mice and humans (4). Furthermore, we recently reported the importance of ISG15 in regulating macrophage activity (35), indicating that ISG15 is a crucial molecule in the regulation of the immune response. Additional studies to understand the biological functions of unconjugated intracellular and/or extracellular ISG15 and of ISGylation may help our knowledge of innate immunity and on how viruses have developed strategies to subvert it.
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Figure legends

Figure 1. VACV E3 protein binds to ISG15 from human and murine origin and blocks ISGylation. A. The C-terminal domain of E3 interacts with human and mouse ISG15. 293T cells were transfected with the indicated plasmids; GST, GST-hISG15 (human origin) or GST-mISG15 (murine origin) and cotransfected with B/NS1 from influenza B/Yamagata/88 virus (Yam88/NS1) or E3 protein from VACV (Western Reserve strain), or with last 26 C-terminal amino acids of E3 (VVE3LΔ83N), or with the first 83 N-terminal amino acids of E3 (VVE3LΔ26C). Cell lysates were subjected to GST pull-down and Western blot analysis of precipitated proteins was performed using GST-, NS1/B- and E3-specific antibodies. Molecular mass markers (kDa) are shown to the right. Three independent experiments were conducted and a representative image is shown. B-C. E3 inhibits human and mouse ISGylation. 293T cells were transfected with the indicated plasmids and then 24 hours later cell lysates were analyzed by Western blotting for V5-ISG15 conjugation using a V5 tag-specific antibody. The expression of the different E3 domains was analyzed by Western blot using a specific antibody against E3. Tubulin was used as loading control. B. Human E1, E2, E3, and V5-hISG15 plasmids were
cotransfected with plasmids encoding B/NS1 or E3 protein from VACV (WR strain), either with
the last 26 C-terminal amino acids of E3 (VVE3Δ83N) or with the first 83 N-terminal amino
acids of E3 (VVE3Δ26C). The concentration of conjugation machinery plasmids was kept
constant, while the relative amounts of hISG15 plasmid were lowered and hISG15 was replaced
by B/NS1. In the line indicated as ISG15-AA, a control of non-conjugated ISG15 (v5-hISG15-
AA) was transfected with human E1, E2, E3. Three independent experiments were performed and
a representative image is shown. C. Murine E1, E2, E3, and V5-hISG15 plasmids were
cotransfected with plasmids encoding B/NS1 or E3, either with the last 26 C-terminal amino
acids of E3 or with the first 83 N-terminal amino acids of E3. In the line indicated as ISG15-AA,
a control of non-conjugated ISG15 (v5-mISG15-AA) was transfected with murine E1, E2, E3.
Three independent experiments were performed and a representative image is shown.

Figure 2. Effect of ISG15 and ISGylation on virus growth and cytotoxicity after infection of
MEFs with VACV or VVΔE3L viruses. A. ISG15−/−, OTU or ISG15+/+ were mock-infected or
infected at 0.1 PFU/cell with VACV (WR) or VVΔE3L. At different times postinfection, cells
were harvested and virus yields were determined by plaque assay for VACV or by immunostaining
for VVΔE3L. B. Cellular viability of ISG15−/−, OTU or ISG15+/+ cells infected with VACV
(WR) or VVΔE3L virus at the indicated multiplicity of infection (MOI) from 0.01 to 10 PFU/cell.
24 hours post-infection (hpi), the medium was removed and cytolysis was determined by crystal
violet staining. The percentage of viable cells after infection was calculated assuming the survival
rate of uninfected cells to be 100%. Three independent experiments were carried out and a
representative experiment is shown. Error bars indicate the standard deviation of the mean.
Student’s t-test was performed to determine the P value. (**, P<0.05; *, P<0.01).
Figure 3. A decrease in the ISGylation levels does not vary VACV pathogenesis. A. Transgenic OTU (ISGylation deficient, grey circles) or wild type mice (white circles) were infected intranasally with $10^5$ PFU/mouse or $5 \times 10^5$ PFU/mouse or $5 \times 10^6$ PFU/mouse of VACV (WR strain). Infected mice were weighed daily and mean percentage weight loss of each group ($n = 12$) was compared with the weight immediately before the infection (uppers panels). The lower panels show survival rate. Two independent experiments were carried out and a representative experiment is shown. Error bars indicate the standard deviation of the mean. Student's t-test was performed to determine the $P$ value. (**, $P<0.05$; *, $P<0.01$). B. VVΔE3L infection is attenuated in the OTU mice. ISG15−/− (black circles), or transgenic OTU (grey circles) or WT mice (white circles) were infected by the intranasal route with $10^8$ PFU/mouse. Infected mice were weighed individually daily and mean percentage weight loss of each group ($n = 12$) was compared with the weight immediately prior to infection (uppers panels). The lower panels show survival rate. Two independent experiments were carried out and a representative experiment is shown. Error bars indicate the standard deviation of the mean. Student's t-test was performed to determine the $P$ value. (**, $P<0.05$; *, $P<0.01$). C. Viral replication of different VACV strains in infected ISG15+/+, ISG15−/− and OTU mice. At 24 hpi lung homogenates were titrated by plaque assay in BSC40 (for WR) or by immunostaining in BHK-21 cells (for VVΔE3L). Three independent experiments were carried out and a representative experiment is shown. Results represent the mean ± SD for samples from individual samples of 6 mice/day/group. Student’s t-test was performed to determine the $P$ value. (*, $P<0.01$). The dotted line represented the detection limit.

Figure 4. Free ISG15 is essential for controlling the pro inflammatory response after infection of VVΔE3L. A. Histopathology of lungs from ISG15−/−, OTU and ISG15+/+ mice intranasally infected with VVΔE3L ($10^8$ PFU/mouse). At 24hpi infected-lungs were resected,
sectioned and stained with hematoxilin and eosin. Three independent experiments were performed using n=3, for each group of animals representative fields of the 9 histology analyzed are shown at a magnification of 100X. B. Western blot of ISG15 in lung homogenates from ISG15−/−, OTU and ISG15+/+ mice intranasally infected for 24 hours with VACV or VVΔE3L (10⁸ PFU/mouse). Each sample represents pools from 6 mice per group. On the right, the molecular weight of the proteins in kilodaltons is indicated. Actin levels showed that the same amount of protein was loaded on the gel. Three independent experiments were carried out and a representative image is shown. C. IL6 levels in serum of ISG15−/− (black bars), or OTU (grey bars) or WT mice (white bars) infected with VVΔE3L (10⁸ PFU/mouse) were measured by ELISA from serum collected at 3 hpi. Three independent experiments were carried out and a representative experiment is shown. Results represent the mean ± SD of pooled samples from 6 mice. Student’s t-test was performed to determine the P value (*, P<0.01). D. ISG15−/− and ISG15+/+ mice were inoculated intraperitoneally with polyI:C (10 mg/Kg), LPS (15 mg/Kg) and Zymosan (15 mg/Kg) and the survival curves of all the groups (n=12) were represented with the time. Two independent experiments were carried out and a representative experiment is shown. E. IL6 levels in serum of ISG15−/− (black bars), or WT mice (white bars) inoculated intraperitoneally with polyI:C (10 mg/Kg), LPS (15 mg/Kg) and Zymosan (15 mg/Kg) were measured by ELISA from serum collected at 3 hpi. Three independent experiments were carried out and a representative experiment is shown. Results represent the mean ± SD of pooled samples from 6 mice. Student’s t-test was performed to determine the P value (**, P<0.05; *P<0.01.

References

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Figure 1
Figure 2

A
Viral titre (pfu/ml) /x 10^{-4}
VVDE3L
5
10
15
20
25
30
35
ISG15-/-
WT
OTU

B
Viral titre (pfu/ml) /x 10^{-5}

C
% Cellular viability

D
% Cellular viability

% Cellular viability

24 hrs post-infection
**Figure 3**

A. **VACV**

- 5x10^6 pfu/mouse
- 5x10^7 pfu/mouse
- 10^5 pfu/mouse

B. **VVΔE3L**

- 10^6 pfu/mouse
- 10^5 pfu/mouse

C. **Virus titers**

- ISG15-/-
- ISG15+/+
- OTU

**Days post-inoculation**

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**% Survival**

- Initial weight

**% Initial Weight**

- N= 12

**Virus titers (pfu/g lung tissue x 10^5)**

- WR ΔE3L
- WR ΔE3L
- WR ΔE3L

**Survival**

- N= 12

**Days post-inoculation**

- N= 12

**Virus titers**

- N= 12

**Survival**

- N= 12

**Days post-inoculation**

- N= 12

**Virus titers**

- N= 12

**Survival**

- N= 12

**Days post-inoculation**

- N= 12

**Virus titers**

- N= 12

**Survival**

- N= 12

**Days post-inoculation**

- N= 12

**Virus titers**

- N= 12

**Survival**
Figure 4

ISG15-/- IL-6 (pg/ml) in serum
200
400
600
800
1000
1200
1400
1600
1800
ISG15+/+ 0
OTU

mouse ISG15 conjugates

Actin

ISG15+/+ ISG15-/- OTU

MW (Kda)

-120
-85
-46

ISG15+/+
ISG15-/-

* *
*
**
*
*

0
25
50
75
100
0
25
50
75
100

Days post-inoculation

D

% Survival

ISG15-/-
ISG15+/+

pIC

LPS

Zymosan

E

% Survival

IL-6 (pg/ml) in serum

ISG15-/-
ISG15+/+

pIC LPS Zymosan

Figure 4
Correction for Eduardo-Correia et al., ISG15 Is Counteracted by Vaccinia Virus E3 Protein and Controls the Proinflammatory Response against Viral Infection

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Volume 88, no. 4, p. 2312–2318, 2014. Page 2317, Acknowledgments: Paragraph 2 should be replaced with the following.

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