Autoimmune regulator (AIRE) contributes to Dectin-1–induced TNF-α production and complexes with caspase recruitment domain–containing protein 9 (CARD9), spleen tyrosine kinase (Syk), and Dectin-1

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Background: Autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) syndrome is a complex immunologic disease caused by mutation of the autoimmune regulator (AIRE) gene. Autoimmunity in patients with APECED syndrome has been shown to result from deficiency of AIRE function in transcriptional regulation of thymic peripheral tissue antigens, which leads to defective T-cell negative selection. Candidal susceptibility in patients with APECED syndrome is thought to result from aberrant adaptive immunity.

Objective: To determine whether AIRE could function in anticandidal innate immune signaling, we investigated an extrathymic role for AIRE in the immune recognition of β-glucan through the Dectin-1 pathway, which is required for defense against *Candida* species.

Methods: Innate immune signaling through the Dectin-1 pathway was assessed in both PBMCs from patients with APECED syndrome and a monocytic cell line. Subcellular localization of AIRE was assessed by using confocal microscopy.

Results: PBMCs from patients with APECED syndrome had reduced TNF-α responses after Dectin-1 ligation but in part used a Raf-1–mediated pathway to preserve function. In the THP-1 human monocytic cell line, reducing AIRE expression resulted in significantly decreased TNF-α release after Dectin-1 ligation. AIRE formed a transient complex with the known Dectin-1 pathway components phosphorylated spleen tyrosine kinase and caspase recruitment domain–containing protein 9 after receptor ligation and localized with Dectin-1 at the cell membrane.

Conclusion: AIRE can participate in the Dectin-1 signaling pathway, indicating a novel extrathymic role for AIRE and a defect that likely contributes to fungal susceptibility in patients with APECED syndrome. (J Allergy Clin Immunol 2012;129:464-72.)

Key words: *Primary immunodeficiency, innate immunity, chronic mucocutaneous candidiasis, monocytes*

The human primary immunodeficiency disease autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) syndrome is characterized by autoimmunity and chronic mucocutaneous candidiasis (CMC) arising from mutation of the autoimmune regulator (*AIRE*) gene.[1] The AIRE protein is known to localize to the nucleus of medullary thymic epithelial cells and has been shown to be critically involved in the global editing of the T-cell repertoire. As such, the links between *AIRE* mutation and autoimmunity have been somewhat well characterized.[2]

The immune mechanisms underlying candidal susceptibility in patients with APECED syndrome, however, is less clear. Existing data have emphasized aberrant adaptive immunity, including the role of AIRE as a transcriptional mediator ensuring appropriate T-cell repertoire,[2] as well as the development of autoantibodies directed against key antifungal cytokines.[3,4] In addition to expression in thymic epithelial cells, however, AIRE is also constitutively expressed in monocytes and myeloid dendritic cells,[5] thus raising the possibility of a role for AIRE in innate

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Abbreviations used
AIRE: Autoimmune regulator
APECED: Autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy
CARD9: Caspase recruitment domain–containing protein 9
CMC: Chronic mucocutaneous candidiasis
DAPI: 4′,6-Diamidino-2-phenylindole
FACS: Fluorescence-activated cell sorting
PMA: Phorbol 12-myristate 13-acetate
shRNA: Short hairpin RNA
Syk: Spleen tyrosine kinase
TLR: Toll-like receptor
WGA: Wheat germ agglutinin

immunity that is functionally distinct from its role in T-cell maturation.

Candida species are recognized by a variety of innate immune receptors, including the transmembrane c-type lectin β-glucan receptor Dectin-1.[6] Dectin-1 has gained attention because of its involvement in T_H17 cell development and anti–*Candida* species defense.[7,8] In human subjects genetic alteration in Dectin-1 was reported to decrease receptor function and was correlated with CMC.[9] Furthermore, human mutation of caspase recruitment domain–containing protein 9 (CARD9), which is a critical Dectin-1 adaptor, has also been identified in patients with CMC.[10] Thus, the Dectin-1 signaling pathway is essential for anticandidal defense.

Dectin-1 ligation recruits spleen tyrosine kinase (Syk) and subsequently a CARD9-containing complex that ultimately activates the inflammatory response.[8] Mechanisms linking Dectin-1 to the CARD9-containing complex and downstream transcriptional effectors are unclear. An additional pathway induced after Dectin-1 ligation, however, depends on the serine/threonine kinase Raf-1, which integrates with Syk before downstream effector function.[11]

We thus sought to explore mechanisms by which AIRE might directly participate in signaling during the monocytic response to *Candida* species. Monocytic cells from patients with APECED syndrome are known to have an abnormal response to *Candida* species antigens, as measured by cytokine production and protein phosphorylation.[12,13] However, a direct mechanistic role for AIRE in this innate response has not been evaluated. Because AIRE contains its own CARD domain[14] and subcellular localization studies of fluorescent AIRE have demonstrated its presence in the cytosol,[15] we hypothesized that AIRE might function specifically in the Dectin-1 response to *Candida* species within the cell cytoplasm. We found that AIRE deficiency results in reduced β-glucan–induced TNF-α release in both patients' cells and an *in vitro* model of AIRE deficiency. Furthermore, we found AIRE in the cytoplasm of THP-1 monocytes in an activation-induced complex containing Dectin-1, phosphorylated Syk, and CARD9 that might function to enable optimal TNF-α release. This demonstrates a new extrathymic innate immune role for AIRE and a novel signaling complex that is likely defective in patients with and might contribute to the human primary immunodeficiency APECED syndrome.

METHODS

Cells from patients with APECED syndrome

Whole blood obtained from patients with APECED syndrome or healthy control subjects was processed and shipped according to protocols approved by the institutional review boards for the protection of human subjects or ethics committees of the respective participating institution. Patients had the following *AIRE* mutations: (1) 205-208dupCACG, (2) 994del[13], (1) Asp70fsX148, (2) R257X, (1) compound heterozygote,[16] and (1) 967_979del14bp. PBMCs were isolated within 48 hours of blood being drawn.

Cells, antibodies, and reagents

The human monocytic leukemia THP-1 cell line was used and, where described, was differentiated as previously reported[17] or was transfected with 0.5 μg of Myc-tagged Dectin-1 (RC221893; OriGene, Rockville, Md) by using nucleus-targeted electroporation (Nucleofector, Amaxa, program U-001; Lonza, Cologne, Germany) and nucleofection reagent V. Anti-human antibodies used were as follows: Dectin-1 (goat pAb N-16, Western Blot and immunoprecipitation; Santa Cruz Biotechnology, Santa Cruz, Calif), c-myc (mAb 9E10, immunofluorescence; Abcam, Cambridge, United Kingdom), AIRE-1 (goat pAb D-17 Western Blot and immunoprecipitation [in Fig 3, *D*], Santa Cruz Biotechnology; rabbit pAb H-300 for immunoprecipitation [in Fig 3, *A*], immunofluorescence, Santa Cruz Biotechnology; and mAb 6.1, a kind gift from Dr Part Peterson, University of Tampere, for fluorescence-activated cell sorting [FACS]), CARD9 (goat pAb T-17, Santa Cruz Biotechnology), Syk (mouse mAb 4D10, Santa Cruz Biotechnology), and phosphorylated Syk (pY525 + pY526, rabbit pAb EP575[2]Y, Abcam).

AIRE short hairpin RNA and FACS

THP-1 cells were transduced with lentivirus-containing AIRE or nonspecific control short hairpin RNA (shRNA; sc-37669-V and sc-108080, respectively; Santa Cruz Biotechnology) in vectors containing puromycin resistance to enable selection and maintenance. AIRE levels were assessed by using intracellular FACS in resulting cell lines with isotype control or mAb 6.1 followed by fluorescein isothiocyanate–conjugated goat anti-mouse antibody (Sigma, St Louis, Mo) or by using Western blot analysis.

Stimulation and TNF-α release

PBMCs or differentiated THP-1 cells were stimulated with either 10 μg/mL curdlan (Dectin-1), 2 μg/mL R848 (Toll-like receptor [TLR] 7/8, differentiated THP-1 cells only), or phorbol 12-myristate 13-acetate (PMA)/ionomycin (100 nmol/L and 10 nmol/L, respectively, PBMCs only) for 24 hours, and TNF-α release was measured, as previously described.[18]

Analysis of AIRE-interacting proteins

Undifferentiated THP-1 cells (1.5×10^7) were unstimulated or stimulated with curdlan (10 μg/mL) for 1, 10, or 60 minutes or 12 hours; lysed; and subjected to centrifugation. Selected proteins were immunoprecipitated and evaluated by means of Western blot analysis with 4% to 12% SDS containing Bis-Tris gels (Invitrogen, Carlsbad, Calif), as previously specified.[19] Because of the size of individual experiments, different coimmunoprecipitations were not performed on the same day. Band densities were measured with ImageJ software (National Institutes of Health, Bethesda, Md) and normalized to the unstimulated condition. The heavy chain was specifically and intentionally cropped out in the AIRE blot shown in Fig 3, *D*.

Confocal microscopy

Resting or curdlan-stimulated undifferentiated THP-1 cells were prepared for confocal microscopy, as previously described,[19] and stained with fluorescein isothiocyanate–conjugated wheat germ agglutinin (WGA), 4′,6-diamidino-2-phenylindole (DAPI), anti–AIRE H-300, anti-myc 9E10, or preimmune isotype (rabbit or mouse, Novozymes, Bagsvaerd, Denmark). For the representative images shown in Fig 5, radial intensity plots were generated with Volocity software (PerkinElmer, Waltham, Mass), and graphs depict channel-specific mean fluorescence intensities along concentric circles emanating from the centroid of each cell expressed as percentages of each respective maximum intensity (conversion factor = 0.0929 μm per pixel).

Assessment of AIRE at the cell membrane with WGA or Dectin-1 or in the nucleus was measured as AIRE colocalized with the respective fluorescent signal by using Volocity, as previously described.[20] Subcellular percentages of AIRE were defined as follows:

$$100\times(\text{Subcellular AIRE area}/\text{Total cellular AIRE area}).$$

Cells were analyzed individually and used to generate means ± SDs.

Statistical analysis

All experiments were performed in at least triplicate, and results were averaged, where specified. Mean data were compared with the nonparametric 2-tailed Mann-Whitney *U* test and Prism software (GraphPad Software, Inc, La Jolla, Calif).

RESULTS

Evaluation of Dectin-1 signaling in PBMCs and THP-1 monocytic cells from patients with APECED syndrome

Given the importance of the Dectin-1 pathway in human anticandidal defense, we evaluated PBMCs from healthy control donors and patients with APECED and *AIRE* mutation for their ability to produce TNF-α as an end point functional readout for Dectin-1 signaling by using the Dectin-1–specific ligand curdlan.[21] No patients had monocytopenia or monocytosis before analysis and not all had CMC. After 24 hours of curdlan stimulation of PBMCs, patients' cell supernatants contained approximately one third less TNF-α when compared with that seen in cells of healthy control donors ($P = .05$, Fig 1).

Because a role for Raf-1 in the Dectin-1 signaling pathway has been described[11] and Raf-1 functions independently of Syk, we postulated that at least some function in the Dectin-1 pathway in patients' PBMCs was Raf-1 mediated. To test this hypothesis, patients' cells or control cells were preincubated with the Raf-1 inhibitor GW5074[22] before curdlan stimulation. GW5074 pretreatment further reduced curdlan-induced TNF-α release in patients' PBMCs compared with those seen in control PBMCs (Fig E1, *A* and *B*, in this article's Online Repository at www.jacionline.org). The inhibitor also reduced the Dectin-1 response in control cells (contrast curdlan-treated control subjects in Fig 1 and Fig E1, *A*), as would be expected given the reported role for Raf-1 in this pathway. However, there was no preferential effect of GW5074 in patients' cells compared with control cells after PMA/ionomycin stimulation, thus demonstrating some specificity for the Dectin-1 pathway (see Fig E1, *B*). Because the initial experiments with patients' cells did reveal off-target effects of the concentration of inhibitor used on overall TNF-α release in PMA/ionomycin–stimulated control cells, the relative effect of the inhibitor on the Dectin-1 pathway was evaluated and persisted in dose-titration experiments using control donor cells (see Fig E1, *C*). Although PMA/ionomycin is a relatively nonspecific stimulus, GW5074 also did not inhibit the response to R848 (TLR7/8 ligand) in control donors' cells (see Fig E1, *C*). This further suggested the specificity of inhibitor function for the Dectin-1 pathway. In total, the further reduction in TNF-α release from patients' PBMCs after GW5074 treatment implicates the Raf-1 signal as an independent pathway from that of AIRE function downstream of Dectin-1 and suggests that cells from patients with APECED syndrome have an inherent defect in the Dectin-1/Syk arm of the pathway.

To further evaluate a mechanistic role for AIRE in Dectin-1 function, we created an AIRE-deficient THP-1 monocytic cell line using lentivirus-transduced AIRE-specific shRNA. As a control, a THP-1 cell line was generated by transducing nonspecific shRNA. The AIRE shRNA–expressing cells contained almost a one-half log reduction in the amount of AIRE protein, as demonstrated by means of FACS (Fig 2, *A*), and quantities that were barely appreciable in Western blot analysis (Fig 2, *B*). Furthermore, the degree of AIRE knockdown did not appreciably change over time (not shown) or in response to Dectin-1 ligation (Fig 2, *B*). These 2 cell lines also proliferated similarly with comparable viability (not shown), indicating that AIRE deficiency did not create global cellular defects in the THP-1 cell line. These cell lines were then stimulated with curdlan to enable evaluation of the role of AIRE in curdlan-induced TNF-α release. To obtain enhanced and appreciable amounts of TNF-α from the THP-1 cells to quantify in ELISA, however, they were first differentiated with PMA. On subsequent stimulation with curdlan, there was significantly less TNF-α release from the AIRE-deficient cells (Fig 2, *C*), recapitulating the patient sample–based observations. TLR7/8 pathway function was analyzed to determine whether this difference was inherent to Dectin-1 stimulation and not a feature of overall hyporesponsiveness of this cell line. There was no measurable difference in TNF-α release between differentiated AIRE-deficient and control THP-1 cells after TLR7/8 ligation with R848, suggesting specificity of AIRE function in the Dectin-1 pathway.

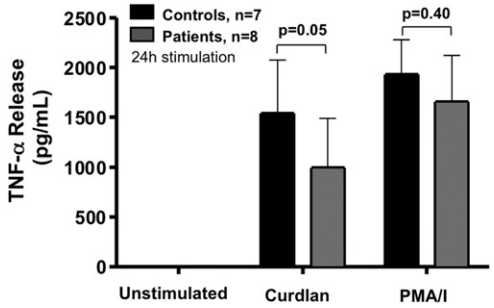

FIG 1. TNF-α release by PBMCs from patients with APECED syndrome after specific ligation of Dectin-1. PBMCs from patients with APECED syndrome or control donors were stimulated with either the nonspecific agonist PMA/ionomycin *(PMA/I)* or the Dectin-1–specific agonist curdlan. TNF-α in supernatant was assessed by using ELISA. *P* values compare TNF-α release from patients and control subjects within either treatment.

AIRE associates with Dectin-1 signaling components

To further define a role for AIRE in the Dectin-1 pathway, using the THP-1 cell line, we evaluated AIRE immunoprecipitates from Dectin-1–stimulated cells for proteins known to be involved in the Dectin-1 signaling pathway: Dectin-1, Syk, and CARD9.[23,24] We chose a shorter time frame of stimulation than that used for the TNF-α release experiments because of the rapid nature of upstream signal generation after innate immunoreceptor ligation. In coimmunoprecipitation experiments trace constitutive associations between AIRE and Syk (Fig 3, *A* and *D*), Dectin-1 and AIRE (Fig 3, *B*), and CARD9 and AIRE (Fig 3, *C*) were identified in unstimulated cells. After 1 minute of curdlan stimulation, however, the association of AIRE with Syk, as well as Dectin-1 and CARD9 with AIRE, increased (Fig 3) and remained at those levels for at least 10 minutes. The association between AIRE

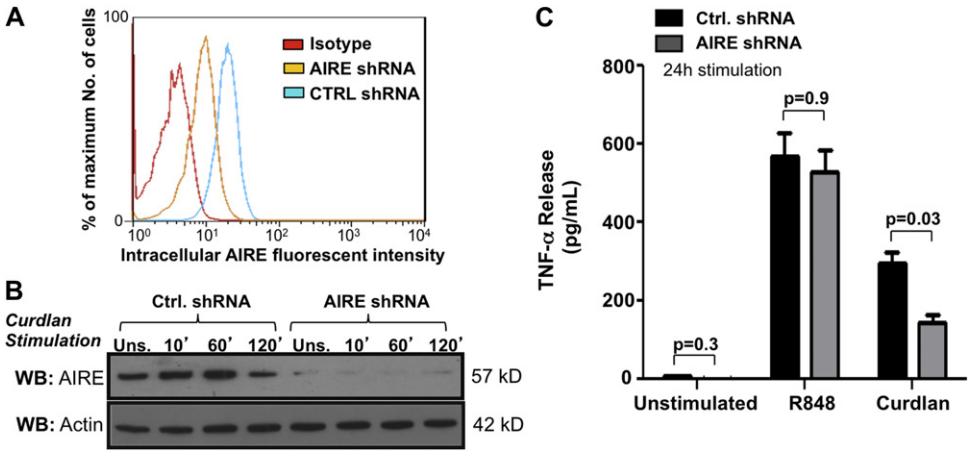

FIG 2. TNF-α release in differentiated AIRE-deficient THP-1 cells. **A,** Intracellular FACS for AIRE comparing THP-1 cells containing control (blue) or AIRE (orange) shRNA. **B,** Western blot analysis of AIRE in control or AIRE shRNA–containing cells with or without curdlan stimulation. *Uns.*, Unstimulated. **C,** Mean TNF-α release in response to 24 hours of incubation with media alone *(Unstimulated)*, curdlan, or R848 from 4 independent experiments with differentiated THP-1 cells.

and Syk was maximal at 1 hour (approximately 2.5-fold increase), as was that between CARD9 and AIRE (approximately 1.7-fold increase). Although the association between Dectin-1 and AIRE was maximal at 1 minute (approximately 1.8-fold increase), all of the associations identified were greater than constitutive levels for at least 60 minutes. Within 12 hours of curdlan stimulation, each of the associations identified had returned to levels found in resting cells (Fig 3, *A-C*). To determine whether the AIRE-containing complex assembled after Dectin-1 ligation showed evidence of participation in active signaling, we evaluated the AIRE immunoprecipitate for phosphorylated Syk (Y525/526)[25] before and after curdlan stimulation. Although the baseline association of Syk and AIRE in THP-1 cells included some phosphorylated Syk, in repeated experiments this was significantly increased by approximately 70% after 10 minutes of curdlan stimulation in a fixed number of cells (Fig 3, *D*). Thus, stimulation with curdlan induced a stable but transient complex in THP-1 cells involving the receptor Dectin-1, activated Syk, CARD9, and AIRE.

Subcellular localization of AIRE with Dectin-1 in response to curdlan stimulation

Because Dectin-1 is a transmembrane receptor and AIRE is widely accepted as a nuclear protein, the spatial dynamics of this signaling complex formation were unclear. Because our immunoprecipitation experiments used cell lysates that had been centrifuged to clear most nuclear material, the possibility of AIRE in the cell cytoplasm as part of a signaling complex was raised. Thus we hypothesized that at least some AIRE could localize to the cell membrane after curdlan stimulation to associate with the Dectin-1 receptor. To initially evaluate the localization of AIRE relative to this signaling complex, we performed fixed-cell confocal microscopy using THP-1 cells either unstimulated or stimulated with curdlan for 10 and 60 minutes. We first evaluated whether AIRE could localize to the cell membrane after activation and thus defined the cell membrane with fluorescently labeled WGA, which binds cell-surface lectins. Cells were then stained for AIRE, and their nuclei were identified with DAPI. Fluorescent signal for AIRE was specific, as demonstrated by comparison with cells stained with preimmune rabbit IgG (see Fig E2 in this article's Online Repository at www.jacionline.org). In unstimulated cells the amount of AIRE not colocalized with the nucleus (ie, in the cytoplasm) or that specifically colocalized with the cell membrane (nonnuclear WGA) was low (Fig 4, *A* and *B*). However, analysis of multiple cells showed that upon curdlan stimulation for 10 minutes, the percentage of total AIRE present in the cytoplasm (Fig 4, *C*) was increased relative to that in the nucleus. The fraction of AIRE at the cell membrane, as defined by colocalization with WGA, also increased (Fig 4, *D*) after activation and returned to baseline levels at 60 minutes.

Because AIRE expression at the cell membrane increased after Dectin-1 ligation, we wanted to determine whether AIRE colocalized with Dectin-1 specifically after stimulation. Because of weak detection of Dectin-1 in THP-1 cells by using immunofluorescence, we exogenously expressed myc-tagged Dectin-1 in THP-1 cells so that we could use a specific reagent directed against the myc tag. These cells retained an ability to respond to curdlan and produced approximately twice as much TNF-α after 24 hours of curdlan stimulation relative to that seen in mock-transfected THP-1 cells (data not shown), corresponding to the exogenous expression of additional functional Dectin-1 receptors. These myc-tagged Dectin-1–expressing cells were stained with anti-myc to localize Dectin-1 relative to AIRE and the nucleus. As expected, myc-tagged Dectin-1 was consistently found at the cell surface (Fig 5, *A*). In unstimulated cells AIRE was found in the nucleus but was identified at the cell surface in a punctate pattern with myc-tagged Dectin-1 after curdlan stimulation, most notably at the 10-minute time point (Fig 5, *A*). At high magnification, the cell-surface punctae of myc-tagged Dectin-1 were in part localized with AIRE signal only after curdlan stimulation (Fig 5, *B*). This overall change in localization was directly quantified by using radial intensity measurements that depict the mean fluorescence intensity along concentric circles emanating along a radius from the cell centroid to the plasma membrane 1 pixel at a time. Before curdlan stimulation, only one peak of the AIRE signal was identified, corresponding to the cell nucleus as defined by the peak of DAPI fluorescence. After 10 minutes of curdlan

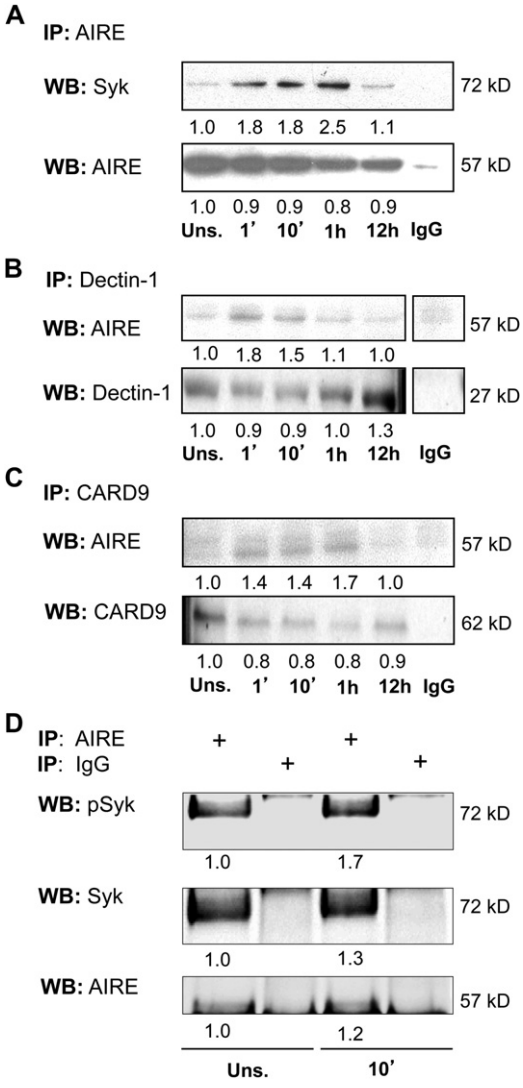

FIG 3. Presence of AIRE in a curdlan-induced Dectin-1–containing complex. Curdlan-stimulated THP-1 cell lysates immunoprecipitated for AIRE (**A** and **D**), Dectin-1 **(B)**, or CARD9 **(C)** antibodies. Western blotting for Syk (Fig 3, *A* and *D*), AIRE (Fig 3, *B* and *C, top*), or phosphorylated Syk (Y525/526) (Fig 3, *D*). Each blot was stripped and reprobed with the immunoprecipitating antibody *(bottom)* to ensure equal immunoprecipitated protein (Fig 3, *A* and *D,* used different AIRE antibodies for IP). Control for nonspecific precipitation is shown with species-appropriate isotype control. Each blot is representative of 3 or more independent experiments. *Uns.*, Unstimulated.

stimulation, however, a second peak of the AIRE signal discrete from the central nuclear signal was apparent at a radius corresponding to the peak fluorescence of the cell membrane–localized myc–Dectin-1 (Fig 5, *C*, red arrow at approximately 65 pixels), demonstrating an activation-induced differential AIRE localization. Formal colocalization analysis of multiple cells further demonstrated that AIRE exhibited significantly increased coincidence with myc–Dectin-1 after 10 minutes of curdlan stimulation (Fig 5, *D*). Increased colocalization was no longer apparent after 1 hour of curdlan stimulation, thus indicating a specific activation-induced recruitment of AIRE to Dectin-1 at the cellular membrane, which corresponds to the changes in AIRE density observed in our biochemical experiments (Fig 3). Overall, these observations identify a novel role for AIRE and suggest new ways in which it could be relevant in patients with the primary immunodeficiency APECED syndrome.

DISCUSSION

The Dectin-1 receptor is integral in coordinating an immune response against *Candida* species and is required for optimal anticandidal defense.[23] Signaling after Dectin-1 ligation uses 2 independent pathways: Syk and Raf-1.[8,11] The former is especially important because impaired responses to β-glucan are found in both human and murine CARD9 deficiency.[8,10] In this work we investigated whether AIRE could participate in the Dectin-1 pathway and potentially contribute to the Dectin-1 response.

In a series of PBMCs from patients with APECED syndrome, we found a blunted response to curdlan, as assessed based on release of TNF-α, that was further reduced on addition of the Raf-1–specific inhibitor GW5074. Given that residual function could be blocked by Raf-1 inhibition, we considered cells from patients with APECED syndrome to be preferentially and inherently defective in the Syk/CARD9 pathway downstream of Dectin-1. There was variability in responses of patients' cells, suggesting that there might be differential impairments attributable to individual mutations or effects of differential CMC morbidity at the time of evaluation. However, because of the relatively small number of patients studied, it was not possible to attribute individual TNF-α release values to specific mutations or other clinical factors. Specific consideration of effects from particular *AIRE* mutations will represent an important future direction in patient-oriented investigation in patients with APECED syndrome. However, we had 2 siblings with the 994del[13] *AIRE* mutation included in the analysis, and they did not have identical responses, which is consistent with prior reports of siblings with the same mutation having variable expressivity in phenotype.[2,26] However, the finding of a reduced TNF-α response to curdlan in patients' cells was specific because the response of these cells to PMA/ionomycin (a nonspecific agonist) was indistinguishable from that seen in control cells.

The results from the analysis of patients' PBMCs were corroborated by the significant difference in TNF-α release between control differentiated THP-1 cells and those with stably reduced AIRE expression, thus further linking this defect to AIRE. The lack of difference in TNF-α release in these transduced THP-1 cells in response to independent pattern-recognition receptor (TLR7/8) ligation, which is not expected to use Syk/CARD9 signaling, additionally suggested specificity for AIRE in the Dectin-1 pathway independent of any more pervasive or nuclear function of AIRE in the cell. Our use of differentiated THP-1 cells to obtain robust TNF-α readings also suggests that differentiation status might affect the Dectin-1/AIRE signaling biology and thus would be an important future direction of investigation to assess developmentally specific AIRE signaling function.

The reduced cytokine production we identified when AIRE expression was abnormal or reduced is consistent with the decreased curdlan responses found in CARD9-deficient murine cells.[8,10] However, the relatively smaller impairment of patients' cells after curdlan stimulation might have AIRE-specific explanations. The Raf-1 pathway remains intact in patients with APECED syndrome, and concurrent infections/inflammation could enhance the Raf-1–dependent response because Raf-1 can be activated through a variety of different innate ligand inputs.[27,28] Alternatively, AIRE might be only enhancing function

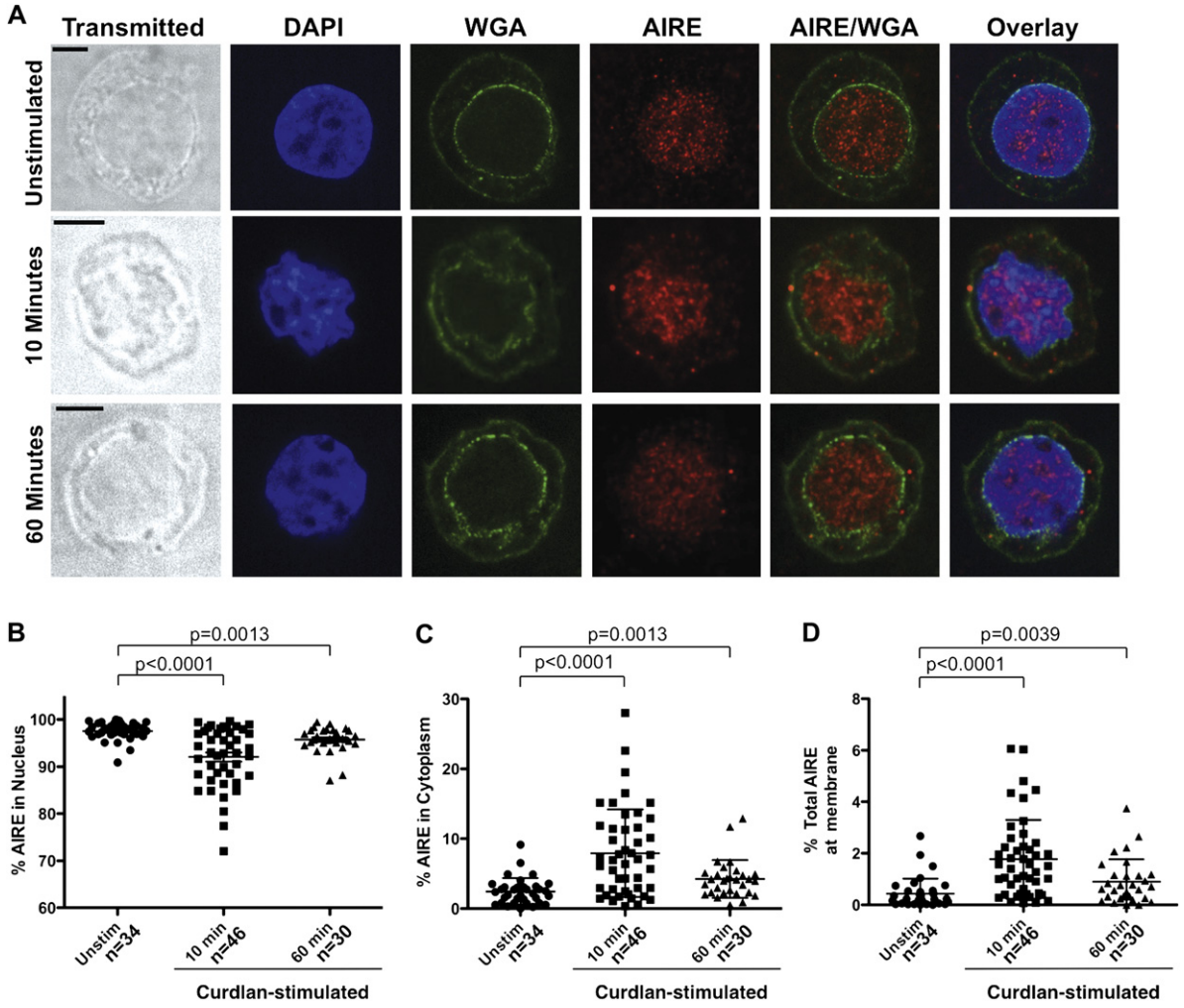

FIG 4. AIRE localizes to the cell membrane after Dectin-1 ligation. **A,** Resting or curdlan-stimulated THP-1 cells stained for membrane (WGA, *green*), AIRE *(red)*, and nucleus (DAPI, *blue*). Images for transmitted light *(left)* and individual or overlayed fluorescent images *(right)* are shown. **B-D,** Quantitation of AIRE signal in the nucleus (Fig 4, *B*), cytoplasm (Fig 4, *C*), or cell membrane (Fig 4, *D*). Each *dot* represents a single cell. *Unstim*, Unstimulated. Data are cumulative from 3 independent experiments. *Scale bar* = 5 μm.

through the Syk/CARD9 pathway and not be absolutely required for it. In this capacity AIRE would represent a facilitator of the CARD9 signal. Functionally, however, our data emphasize a novel role for AIRE in enabling an optimal response to β-glucan.

Our functional experiments predicted that AIRE would contribute directly to the Dectin-1/Syk/CARD9 pathway. Coimmunoprecipitation experiments with THP-1 cells defined a transient, ligation-induced association among AIRE, CARD9, phosphorylated Syk, and Dectin-1, suggesting these complexes are cytoplasmic and dependent on posttranslational modifications for assembly. This was consistent with our observations regarding the subcellular localization of AIRE in THP-1 cells, in which a minor portion of AIRE was found outside of the nucleus and was available to colocalize with Dectin-1 at the cell membrane after curdlan stimulation. Importantly, there was a consistent pool of AIRE (approximately 2.5% of total) that colocalized with the cell membrane or myc–Dectin-1 after curdlan stimulation across independent experiments, further suggesting specific activation-induced recruitment of AIRE to Dectin-1. It should be noted that our microscopy experiments identified the vast majority (>90%) of AIRE to be in the nucleus. Although this suggests that the small proportion of cytoplasmic AIRE is secondary in function to the principal *in vivo* role of AIRE in the nuclear compartment, the novel associations observed for cytoplasmic AIRE suggest it participates in generating a fully functional response downstream of Dectin-1 (Fig 6).

How AIRE might directly participate in signaling remains unclear. AIRE has a CARD domain at its N-terminus[14] that could conceivably facilitate interactions with other CARD-containing proteins, such as CARD9, to enable participation in anticandidal defense. We also found that AIRE associates with phosphorylated Syk after curdlan stimulation, a further suggestion of participation in an active signaling process. Because AIRE-deficient antigen-presenting cells have been shown to have defects in tyrosine kinase activation after exposure to *Candida* species antigens[12] and because Syk is required for Dectin-1 signaling,[24] Syk is a likely candidate to be affected by AIRE deficiency. How AIRE might access Syk for full Dectin-1 function will be an important focus of future studies, including the physical nature of the associations, because AIRE is not otherwise known to interact with

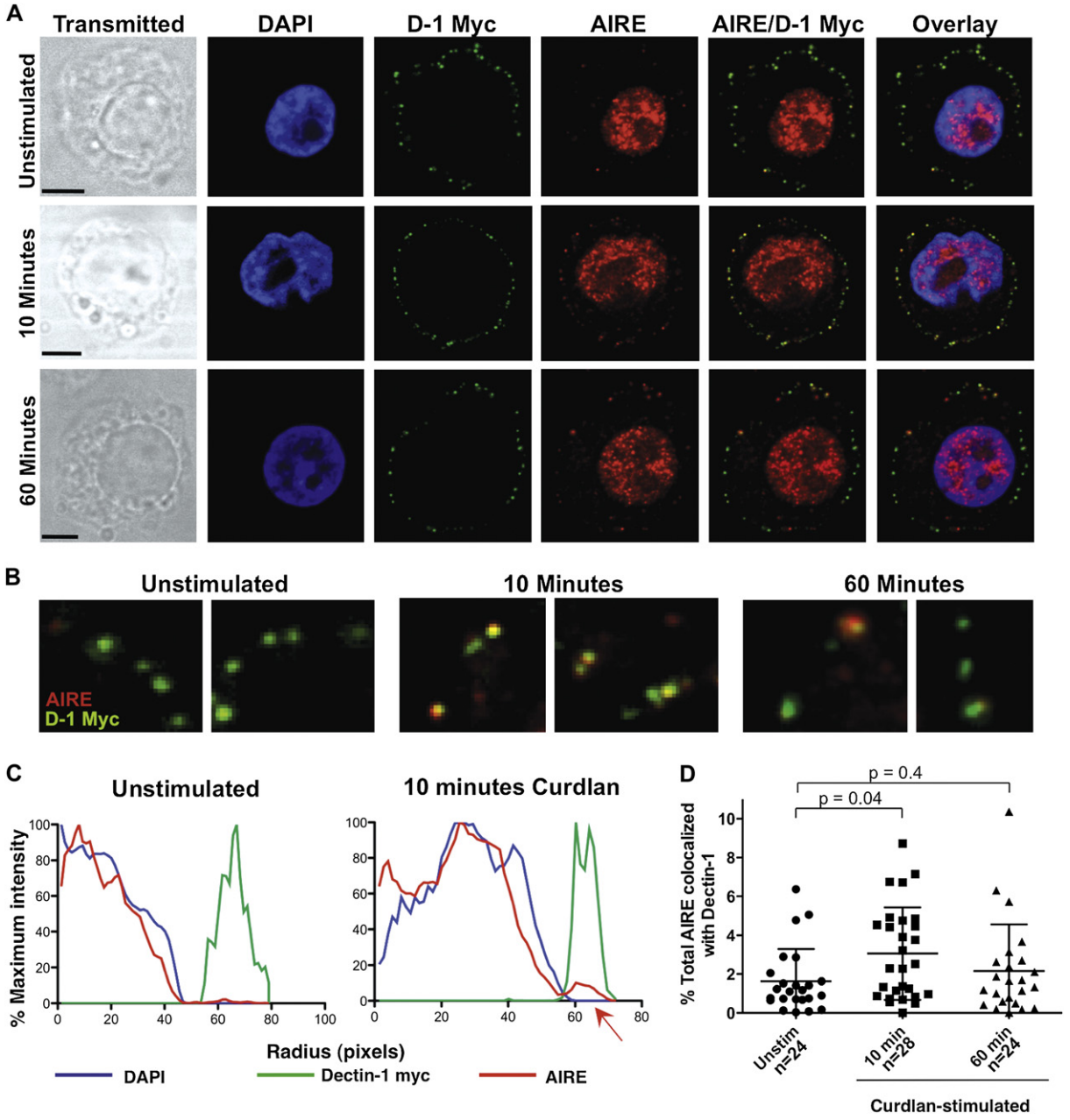

FIG 5. AIRE colocalizes with Dectin-1 in THP-1 cells after Dectin-1 ligation. **A,** Resting or curdlan-stimulated THP-1 cells stained for anti-myc *(green)*, AIRE *(red)*, and nucleus (DAPI). *D-1*, Dectin-1. **B,** Cropped enlargements of Dectin-1–myc/AIRE colocalization in Fig 5, *A*. **C,** Radial intensity plots for each signal. The *red arrow* highlights a second nonnuclear peak of AIRE intensity. **D,** Quantitation of AIRE/Dectin-1–myc colocalization (each *dot* represents a single cell). *Unstim*, Unstimulated. Data are cumulative from 3 independent experiments. *Scale bar* = 5 μm.

cytoplasmic proteins in immune cells. However, we point out that AIRE has also been found to be cytoplasmically expressed in the outer root sheath of hair follicles and associates with keratin-17,[29] which further supports a potentially distinct role for AIRE in this cellular compartment and will represent a departure from studies of other known interacting partners that exist in the nucleus.[30,31] There is also precedent for an immunologically relevant protein with clear primary nuclear functions also having a cytoplasmic role. The transcription factor class II transactivator, another CARD-containing transcriptional protein,[32] has direct cytoplasmic function in HIV-1Pr55Gag processing.[33] Thus, although not unprecedented, we document and propose a nonnuclear role for a primarily nuclear protein. Overall, we hypothesize that the Dectin-1/Syk/CARD9 pathway uses AIRE and is impaired in patients with APECED syndrome, at least in generating a full TNF-α response to fungal β-glucan (Fig 6).

There are multiple potential mechanisms as to how these data might fit into the immunologically complex milieu of CMC. For one, it is possible that functionally redundant pathways might be affected by AIRE deficiency. In particular, a growing appreciation

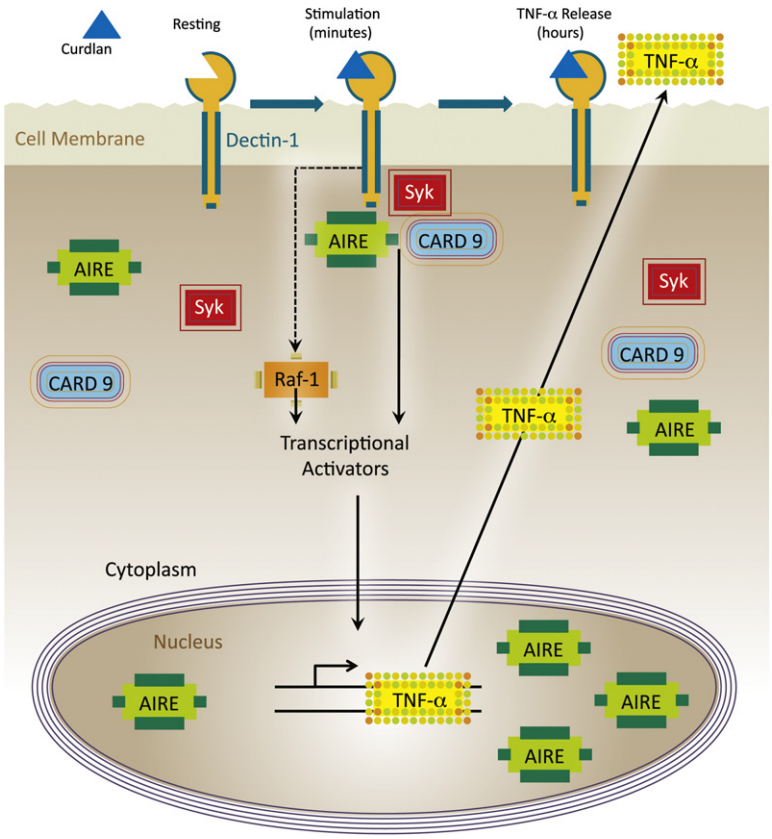

FIG 6. Summary diagram. The model depicts a potential function for AIRE in the cytoplasm by its participation in a newly defined complex containing Dectin-1, Syk, and CARD9 at the cell membrane within minutes of Dectin-1 ligation by curdlan in THP-1 cells. This pathway might act in parallel with the Raf-1 pathway to facilitate downstream signaling, transcriptional upregulation, and release of the proinflammatory cytokine TNF-α.

of Dectin-2–deficient mice suggests that Dectin-2, a lectin distinct from Dectin-1 in that it recognizes α-mannans, participates alongside Dectin-1 in anticandidal defense.[34,35] Because both Dectin-1 and Dectin-2 use similar signaling effectors (Syk/CARD9), further studies regarding how AIRE might be involved in Dectin-2 signaling would be useful to fully discern roles for AIRE in anticandidal responses. Alternatively, reduced Dectin-1 function found in patients with APECED syndrome could lead to insufficient mucosal clearance of *Candida* species through aberrantly low inflammation. In addition, autoantibodies directed against the IL-17 family of cytokines have been recently suggested as the potentially major immunopathogenic mechanism for CMC susceptibility in patients with APECED syndrome.[3,4] Therefore we reason that a chronic candidal burden is needed to generate prolonged IL-17 exposure and thereby break tolerance to facilitate formation of the autoantibodies. We propose that blunted AIRE-mediated Dectin-1 signaling would create a milieu in which reduced Dectin-1–directed anticandidal inflammation and clearance at mucosal surfaces enables a tonic anticandidal response with prolonged (but insufficient) production of IL-17 in response to chronic *Candida* species infection. In the context of AIRE deficiency, this could potentially facilitate the generation of the observed anti–IL-17 autoantibodies.

Our findings and the potential mechanisms discussed emphasize the complex communication between innate and adaptive immunity in the control of *Candida* species.[36,37] They also suggest therapeutic opportunity in patients with APECED syndrome through reducing candidal exposure or enhancing defective innate immunity before the onset of CMC in patients with AIRE mutation. Most importantly, our results demonstrate a previously unappreciated extranuclear role for AIRE in human monocytic cells that is likely to factor into the mechanistically complex susceptibility to CMC in patients with APECED syndrome through impairing function of the Dectin-1 pathway.

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Key messages

- **Patients with APECED syndrome have decreased TNF-α release in response to Dectin-1 ligation.**
- **AIRE might facilitate a role independent of its well-characterized transcriptional abilities to shape the inflammatory response to candidal elements.**
- **AIRE participates in the well-defined signaling complex required for anticandidal defense, including Dectin-1, phosphorylated Syk, and CARD9.**

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METHODS

PBMC stimulation and Raf-1 inhibition

PBMCs from patients and control subjects were preincubated with 76 μm of the Raf-1 inhibitor GW5074 (Sigma) 1 hour before stimulation with PMA/ionomycin or curdlan, which did not affect cell viability as measured by trypan blue exclusion. This concentration was determined in an initial dose titration (data not shown) to inhibit the curdlan response without affecting viability. Because off-target effects were observed, a repeat titration of GW5074 with varying concentrations (vehicle control or 5, 10, or 20 μmol/L) in control PBMCs was performed with the addition of TLR7/8 stimulation to ensure that GW5074 specifically inhibited the Dectin-1 pathway at a concentration that did not affect other activation pathways.

Isotype control staining for immunofluorescence

THP-1 cells were fixed, mounted, and stained with both DAPI (nucleus) and rabbit IgG (Sigma) to define negative staining relative to the AIRE signal shown in Figs 4 and 5. Images were acquired in parallel with those in Figs 4 and 5.

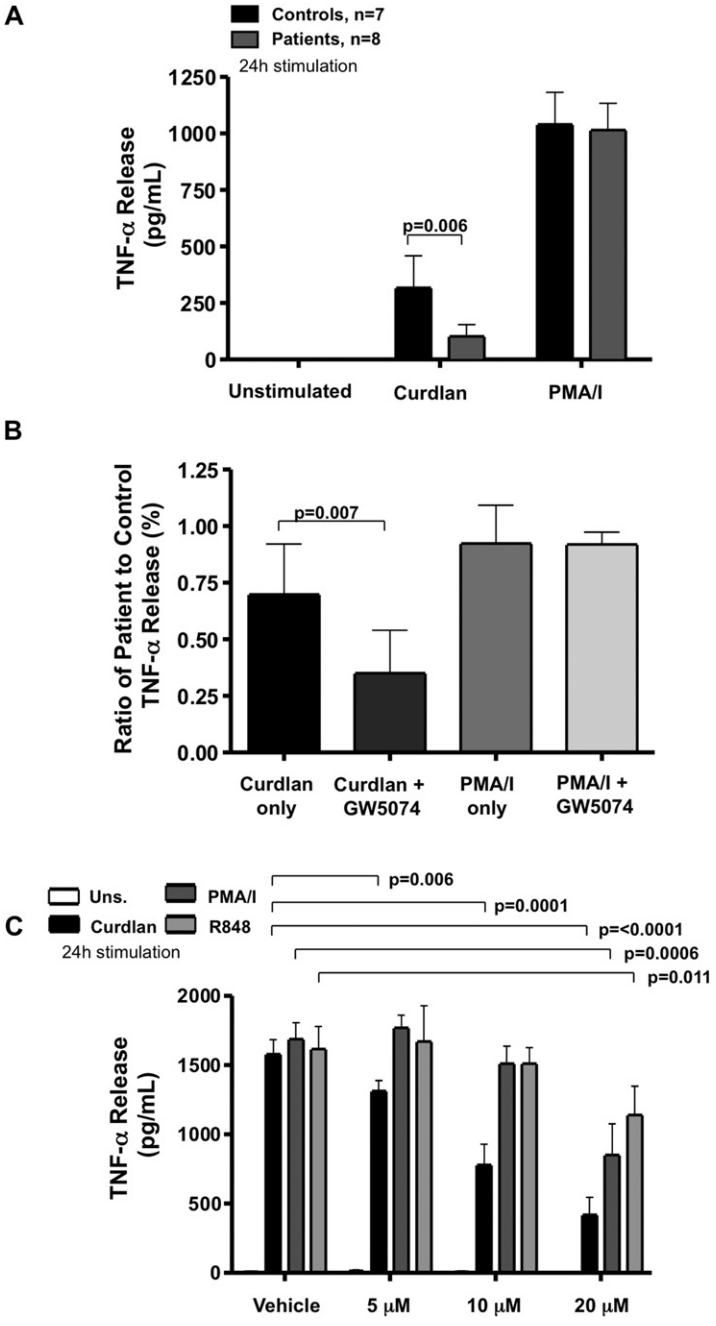

FIG E1. TNF-α production by PBMCs from patients with APECED syndrome after pretreatment with the Raf-1 inhibitor GW5074. **A,** PBMCs from patients with APECED syndrome or control donors were pretreated for 1 hour with 40 μg/mL of the Raf-1 inhibitor GW5074 before incubation with either PMA/ionomycin *(PMA/I)*, curdlan, or media *(Unstimulated)* for 24 hours. TNF-α levels in the supernatant were assessed by means of ELISA. **B,** The same values depicted in Fig E1, *A*, and Fig 1, *A*, are expressed as a ratio between the TNF-α released by patients over that seen in control subjects for each treatment condition. *P* values compare the means of each treatment ratio ± GW5074. **C,** Because of observed off-target effects of GW5074 in experiments on patients' cells, a separate titration was performed with an independent pattern-recognition receptor ligand to ensure specificity. PBMCs from control donors were pretreated for 1 hour with different concentrations of GW5074 and then stimulated with either PMA/ionomycin, R848 (TLR7/8 ligand), or curdlan for 24 hours. TNF-α levels in the supernatant were assessed by means of ELISA. Titration revealed 10 μmol/L as the optimal concentration for GW5074-mediated Raf-1 inhibition. For all graphs shown here, only significant *P* values (<.05) are depicted. *Uns.*, Unstimulated.

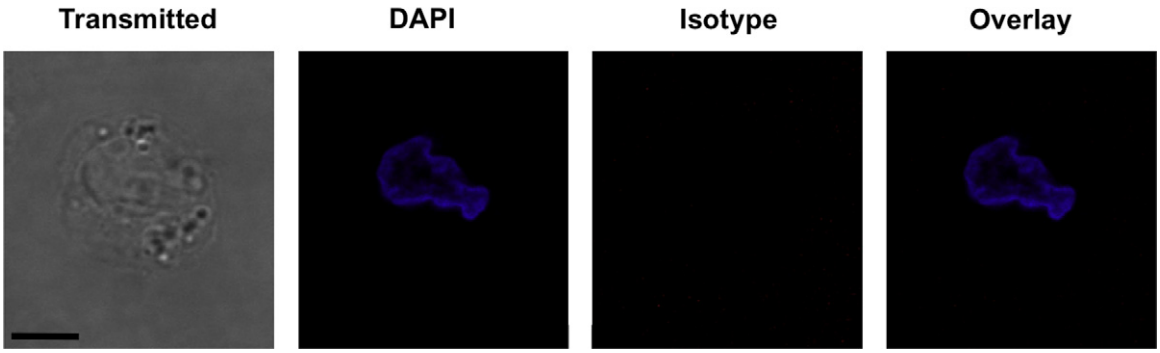

FIG E2. Isotype control staining of THP-1 cells. Representative THP-1 cells stained with both DAPI (nucleus, *blue*) and rabbit IgG *(red)* as a negative control for nonspecific staining. *Overlay* reveals a lack of positive staining and suggests a positive signal for AIRE staining in Figs 4 and 5.