Recycled IL-7 Can Be Delivered to Neighboring T Cells

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*J Immunol* 2015; 194:4698-4704; Prepublished online 13 April 2015; doi: 10.4049/jimmunol.1400560

http://www.jimmunol.org/content/194/10/4698

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**Supplementary Material**

http://www.jimmunol.org/content/suppl/2015/04/11/jimmunol.1400560.DCSupplemental

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Recycled IL-7 Can Be Delivered to Neighboring T Cells

Douglas A. Bazdar,* Magdalena Kalinowska,† Soumya Panigrahi,* and Scott F. Sieg*

IL-7 is a key homeostatic cytokine that provides signals for T cell survival and proliferation in vivo. In this article, we provide evidence that IL-7 utilization is enhanced by a novel mechanism of cytokine “recycling” during which T cells treated with rIL-7 are rapidly induced to express p-STAT5 and are subsequently able to recycle biologically active cytokine for release to neighboring cells in soluble form. Our observations indicate that the ability of cells to recycle IL-7 is dependent on IL-7R α-chain (CD127) and endocytosis, consistent with a model whereby IL-7 is internalized via receptor interactions before recycling. These observations provide evidence of a novel mechanism that enables cells to optimally use IL-7. The Journal of Immunology, 2015, 194: 4698–4704.

The importance of IL-7 in T cell homeostasis is well established. Genetic deficiency of IL-7R α-chain in humans results in immune deficiency characterized by critically low T cell numbers (1). Similarly, mice that lack IL-7 or IL-7Rs have impaired T cell development (2, 3). Adoptive transfer studies have further established that IL-7 plays a critical role in T cell homeostatic proliferation (4, 5) and in vivo administration of IL-7 in humans is a promising tool to reconstitute T cell numbers in lymphopenic settings (6–9). Thus, IL-7 plays a central role in T cell homeostasis, promoting both cellular proliferation and survival of peripheral T cells while also enhancing thymic output.

T cells exposed to IL-7 downmodulate surface receptor (CD127) expression as a consequence of receptor internalization and transcriptional suppression of CD127 mRNA (10). This understanding has led to the concept of “altruistic” T cells, which downmodulate their IL-7Rs once they have obtained sufficient signal, thereby leaving additional cytokine available for other cells (11). In this article, we consider another possible mechanism for optimizing IL-7 availability, in particular, the ability of cells to recycle IL-7 and present the cytokine to neighboring T cells, thereby in effect sharing the cytokine signal.

Unique mechanisms of cytokine presentation have been described for various cytokines including IL-15, IL-2, and IL-6. For IL-15, the α-chain of IL-15 may capture cytokine on one cell and then produce this cytokine in trans to a neighboring cell that expresses the other components of the IL-15R complex (the β-chain and common γ-chain cytokine receptors) (12). In this case, the cell receiving the signal need not express the IL-15R α-chain, whereas the cell delivering the signal requires expression of IL-15R α. IL-2 can be presented in trans by the transfer of the IL-2R α-chain between myeloid dendritic cells and T cells, permitting augmentation of IL-2 signaling in T cells (13), and IL-6 can be transferred via soluble IL-6Rs to membrane-bound gp-130 receptors as a mechanism to induce signaling (14). In this article, we provide evidence that IL-7 can also be presented from one cell to another. Our data suggest that IL-7 interacts with its receptor, is internalized, and at least a portion of cytokine is recycled to the cell surface for release so that it is made available to nearby cells. IL-7 recycling may be an important mechanism that enhances IL-7 utilization.

Materials and Methods

Cells

Whole blood was drawn in heparin-coated tubes from healthy adult volunteers. Volunteers provided written consent. All procedures were approved by the University Hospitals of Cleveland Institutional Review Board. PBMCs were obtained by centrifugation of blood over a Ficoll cushion. Cells were cultured in complete medium consisting of RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO), 0.4% L-glutamine (BioWhittaker), and 0.4% penicillin/streptomycin (BioWhittaker). Purified CD4+ T cells were obtained for some studies by magnetic bead negative selection (Miltenyi) of PBMCs. Cells were >97% pure as judged by flow cytometry.

IL-7 recycling assays

To assess the capacity of cells to transfer IL-7 to neighboring cells, PBMCs, we preincubated purified CD4+ T cells, THP-1, or Jurkat tumor cell lines with rIL-7 generated from Escherichia coli (Cytheris) at various concentrations for 15 min or overnight. Some studies used glycosylated IL-7 produced in human embryonic kidney (HEK) cells (PROSPEC, East Brunswick, NJ). After incubation with IL-7, cells were washed at least twice with 10 ml complete medium and then plated with CFSE-labeled PBMCs. p-STAT5 signaling was detected in CFSE+ cells by flow cytometry after 15 min of coinoculation. In some assays, anti-CD127 (Dendritics) or isotype control Ab was added to cells during the preincubation with rIL-7. Also, inhibitors of endocytosis such as phenylarsine and chlorpromazine were added to cells preincubated with IL-7 in some experiments. In other assays, neutralizing anti–IL-7 Ab (10 µg/ml; R&D Systems) or isotype control Ab was added to the mixed cell cultures. Some assays included acid wash. For these experiments, PBMCs were incubated overnight with rIL-7 (50 ng/ml) and subsequently washed twice with 0.2 M glycine buffer/0.15 M NaCl (pH 3). Cells were then washed twice with PBS. These cells were incubated with CFSE-labeled PBMCs for various times, and both CFSE+ and CFSE− cells were assessed for intracellular p-STAT5 expression by flow cytometry.

Immunocytochemistry, microscopy, and image analysis

PBMCs were incubated for 1 h with rIL-7 (100 ng/ml) at 37°C in RPMI 1640 medium. Cells were washed and cytospin preparations were fixed in 4% paraformaldehyde for 15 min, washed in PBS, permeabilized by 0.5%...
p-STAT5 signaling

Intracellular p-STAT5 was detected by flow cytometry as we previously described (15, 16). In brief, cultured cells were treated with 100 μl of 16% ultrapure methanol-free formaldehyde (Polysciences) for 10 min at 37°C, transferred to polystyrene tubes, washed with PBS, and resuspended in 500 μl cold 90% methanol for 30 min. Cells were washed, stained with Abs for 60 min on ice, washed again, and analyzed with BD LSRII flow cytometer. The Abs used for these assays included anti-rat CD3 PerCP (BD Biosciences), anti-CD4 Pacific blue (BD Pharmingen), CD45RA, and anti-p-STAT5 (Y694) Alexa Fluor 647 or an isotype control (BD Pharmingen).

IL-7 ELISA

Cells that had been preincubated with rIL-7 were washed twice with 10 ml complete medium. These cells were then resuspended in 1 ml and incubated for various times before cell culture supernatants were collected and frozen at −20°C until analyzed by ELISA. Ultrasensitive IL-7 ELISA kits were purchased from R&D Systems. Samples were run in duplicates.

Statistical analyses

Paired data were analyzed with paired Student t tests or with nonparametric Wilcoxon rank sign tests for paired samples (SPSS Software) or with paired Student t tests. Multigroup comparisons were performed with nonparametric one-way ANOVA analyses (SPSS). The p values <0.05 were considered significant.

Results

IL-7 can be transferred between cells to induce p-STAT5 signaling

To assess the potential for cells to present IL-7 to neighboring cells, we incubated PBMCs or purified CD4+ T cells with rIL-7 at various concentrations for 15 min. Cells were then washed and replated with CFSE-labeled PBMCs or purified CD4+ T cells to distinguish between cells that were transferred (CFSE- cells preincubated with IL-7) from cells that had been incubated in medium alone without exogenous IL-7 (CFSE+ responder cells). These cell populations were mixed together for 15 min and p-STAT5 expression was measured. The CFSE+ responder cells gained p-STAT5 signal after only 15 min of coculture with cells previously treated with IL-7. In contrast, cells that had been incubated in a transwell did not demonstrate evidence of p-STAT5 signaling, suggesting that IL-7 from the first culture supernatant was not simply carried over in residual medium after the washes, and that cell proximity may be an important factor in delivery of IL-7 from one cell to another (Fig. 1A, 1B). To confirm that the p-STAT5 signal being observed in responder cells was dependent on IL-7, we performed a similar experiment, but included an anti–IL-7 neutralizing Ab in the assay. Anti–IL-7 Ab almost completely blocked the capacity of IL-7 pulsed cells to induce p-STAT5 signals in responder cells (Fig. 1C, 1D). These data suggest that T cells exposed to IL-7 are able to capture this cytokine and can subsequently deliver it to neighboring cells.

To assess whether CD4+ T cells could mediate this activity directly and to determine a dose response, we incubated purified CD4+ T cells with different concentrations of rIL-7 and then transferred them to purified CD4+CFSE+ responder cells at a 1:1 ratio. The induction of p-STAT5 in responder cells was similar in both CD4+CD45RA+ and CD4+CD45RA− lymphocyte subsets. Transferred cells that had been exposed to rIL-7 for 15 min at concentrations as low as 0.1 ng/ml were able to induce a detectable p-STAT5 signal in responder cells (Supplemental Fig. 1).

CD127 receptor plays an important role in the presentation of IL-7 between cells

We considered the possibility that IL-7R expression may be important for cells to capture IL-7 and to subsequently make it available for other cells. To test this, we incubated cells with anti-CD127 Ab during the 15-min IL-7 pulse. Incubation with anti-CD127 Ab, but not with an isotype control Ab, blocked the capacity of cells to present IL-7 between cells, suggesting that the IL-7R α-chain plays an important role in this process (Fig. 2). Consistent with this finding, CD127+Jurkat, but not CD127−human− THP-1, cells were able...
to mediate delivery of IL-7 to primary T cells (Supplemental Fig. 2). Also, PBMCs that were partially depleted of CD127+ cells with magnetic beads displayed a corresponding reduced capacity to mediate IL-7 presentation to other cells (data not shown). Overall, these data suggest that the IL-7R plays an important role in the ability of cells to present IL-7 to neighboring T cells.

**Endocytosis is important in the ability of cells to deliver IL-7 to neighboring cells**

IL-7Rs are internalized after IL-7 binding, and this process has been linked by other groups to receptor signaling (17). To ascertain whether IL-7R internalization was required for cytokine presentation between cells, we treated cells with phenylarsine, an inhibitor of endocytosis (18, 19), during incubation of cells with rIL-7. Treatment with phenylarsine during IL-7 incubation abrogated the ability of cells to present IL-7 to responder cells (Fig. 3). Similarly, chlorpromazine, an inhibitor of clathrin-coated pit internalization, also reduced IL-7 presentation to responder cells (Fig. 3). At higher concentrations, both reagents inhibited IL-7 signaling in the cells pre-exposed to soluble IL-7, consistent with the importance of internalization of receptors for this activity (17). In separate experiments, phenylarsine was also tested for inhibition of pAKT signaling in PMA-stimulated T cells and was not found to have an effect at the concentrations and incubation periods used in these studies (data not shown). These observations suggest that endocytosis and subsequent trafficking of the internalized complex (IL-7/IL-7R) is required for the subsequent delivery of IL-7 to neighboring cells.

**IL-7 is released as a soluble molecule in cells that present the cytokine to neighboring cells**

Although our initial transwell experiments suggested that cell contact may be important in the cell–cell delivery of IL-7, it is also possible that IL-7 is rerouted to the cell surface, released as a soluble molecule, and simply consumed by CD127+ cells in the lower well, making it less available to cells in the upper wells. To address this possibility, we treated PBMCs with a high concentration of rIL-7 (50 ng/ml) overnight. This overnight incubation resulted in marked downregulation of CD127 receptor to nearly undetectable levels (data not shown), which we reasoned would limit the capacity of these cells to consume IL-7 by reuptake. Cells treated overnight with rIL-7 were washed and then replated in complete medium for 5 min, 30 min, or 2 h. At these time points, either cells or their corresponding supernatants were collected and transferred to responder cells and compared for induction of p-STAT5. At the 5-min time point, transferred supernatants and transferred cells both displayed the capacity to induce p-STAT5 signaling in responder cells (Fig. 4). Increased incubation time, however, resulted in supernatants that largely retained activity and cells that had lost activity. Neutralizing IL-7 Ab blocked the activity observed in the transferred supernatant, indicating that the induction of p-STAT5 in responder cells was largely mediated by IL-7 released in supernatant. These data indicate that IL-7 is rapidly released from IL-7–pulsed cells. The marked reduction in trans presentation observed in the cells over time likely represented their release of IL-7 into supernatants coupled with inefficient reuptake of the cytokine during the incubation period because of the relatively low expression of CD127. Thus, these cells largely “spent” their IL-7 reserve after a short period of incubation and had limited potential to “reload.”

We also used this approach (overnight incubation of cells with IL-7) to confirm that internalized IL-7 was used for presentation to neighboring cells. PBMCs that had been incubated overnight with IL-7 were either washed with complete medium or with acid wash buffer (pH 3). Acid wash was done to remove any residual IL-7 that could have been retained on the cell surface. Despite 2× acid washes, cells pretreated with IL-7 were still able to deliver an IL-7 signal to neighboring cells during both 15-min and 2-h incubations with responder cells, although in one of three samples, there was a clear reduction in activity (Fig. 5). It is possible that some of the reduced activity could be attributed to toxic effects of acid wash. In two samples that were stained with live/dead viability dye after acid washes and 2-h incubation, the acid wash treatment reduced the viability of CD4+ T cells by 29 and 43%. Nonetheless, even if less efficient, it is clear that cells still retained the capacity to deliver IL-7 to neighboring cells despite acid wash.

To directly assess IL-7 release from cells at different time points, PBMCs were preincubated with IL-7 for 15 min, washed, and then replated for additional incubation lasting either 15 min or overnight. Supernatants were collected and assessed for IL-7 by ELISA. These data confirmed that IL-7 was released into supernatants by cells preincubated with IL-7. The amount of cytokine detected in supernatants after replating and overnight incubation was consistently lower than those found at 15 min after replating cells, suggesting that the cytokine continues to be partially consumed by cells during cell culture (Fig. 6A).

Based on these observations, we propose a model whereby IL-7 is internalized by cells and to some extent recycled to the cell surface for delivery to neighboring cells. Previous studies have established that IL-7Rs are blocked from signaling and from internalization by hypertonic solutions (17). Because this process should be reversible upon removal from hypertonicity, we reasoned that cells would be able to capture IL-7 during exposure in hypertonic solution and subsequently internalize and release the cytokine when placed in isotonic conditions. In contrast, we predicted that cells preincubated in hypertonic solution and returned to hypertonic conditions would fail to release IL-7 because the cells would not have the opportunity to internalize the cytokine. To test this possibility, we pulsed cells with IL-7 in hypertonic or isotonic conditions and subsequently washed the cells, replated
the cells in either hypertonic conditions or isotonic conditions, and subsequently collected supernatants after 15-min incubation. Consistent with our prediction, cells that were preincubated with IL-7 in hypertonic sucrose released IL-7 into supernatants when replated in isotonic, but not hypertonic, conditions (Fig. 6B). In contrast, cells preincubated in isotonic conditions and subsequently replated in hypertonic conditions were still capable of releasing IL-7 into supernatants, although this activity was moderately reduced compared with cells incubated only in isotonic conditions. These observations provide evidence that at least some portion of IL-7 can be recycled and released from cells after receptor/cytokine internalization.

To evaluate the activity of recycled IL-7 that had been released into supernatants by cells, we tested four supernatants that had been collected after the cells had been pulsed with IL-7, washed, and replated for 15 min for their ability to induce p-STAT5 signaling. The concentration of IL-7 had been predetermined in these supernatants by ELISA and reported in Fig. 6A. We compared the activity of this recycled IL-7 with our stock of rIL-7. PBMCs were stimulated with rIL-7 from our stock solution to generate a dose–response curve. Cells from the same donor were stimulated with thawed supernatants containing recycled IL-7. The activity of recycled IL-7 closely matched the recombinant stock solution (Fig. 6C). Thus, recycled IL-7 retains its expected biological activity at these specified concentrations.

IL-7 is colocalized with a marker of the exocytic pathway, Rab11, in lymphocytes pulsed with cytokine

The previous observations are consistent with a model whereby IL-7 can be internalized and at least a portion of the cytokine can be rerouted for export and reutilization. To further explore this possibility, we pulsed PBMCs with rIL-7 for 1 h and then examined the cells for evidence of IL-7 colocalization with Rab11, a molecule that identifies recycling endosomes in the cell (20–24). By immunofluorescence microscopy, we found evidence that IL-7 and Rab11 signal show vesicular distribution patterns and colocalization.

<table>
<thead>
<tr>
<th>Condition</th>
<th>p-STAT5</th>
</tr>
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<tbody>
<tr>
<td>medium</td>
<td>43.8</td>
</tr>
<tr>
<td>IL-7</td>
<td>6.1</td>
</tr>
<tr>
<td>IL-7 + DMSO</td>
<td>34.8</td>
</tr>
<tr>
<td>IL-7 + PL (0.13 nM)</td>
<td>37.6</td>
</tr>
<tr>
<td>IL-7 + PL (1.3 nM)</td>
<td>3.7</td>
</tr>
<tr>
<td>IL-7 + CP (50 μM)</td>
<td>5.1</td>
</tr>
<tr>
<td>IL-7 + CP (75 μM)</td>
<td>6.1</td>
</tr>
<tr>
<td>IL-7 + DMSO</td>
<td>55.6</td>
</tr>
<tr>
<td>IL-7</td>
<td>28.5</td>
</tr>
<tr>
<td>IL-7 + PL (0.13 nM)</td>
<td>32.7</td>
</tr>
<tr>
<td>IL-7 + PL (1.3 nM)</td>
<td>58.5</td>
</tr>
<tr>
<td>IL-7 + CP (50 μM)</td>
<td>58.2</td>
</tr>
<tr>
<td>IL-7 + CP (75 μM)</td>
<td>58.2</td>
</tr>
</tbody>
</table>

FIGURE 3. Inhibitors of endocytosis reduce the capacity of cells to transfer IL-7 to neighboring cells. PBMCs were incubated with phenylarsine (PL; 1.3 or 0.13 nM), chlorpromazine (CP; 50 or 75 μM), or DMSO for 15 min and then pulsed with rIL-7 (5 ng/ml) for 15 additional minutes. These cells were washed and mixed with CFSE+ PBMCs in the absence of inhibitors. (A) Representative histograms show p-STAT5 signaling detected by intracellular flow cytometry in responder cells (CFSE+ cells) and in cells used to deliver the IL-7 (CFSE− cells). (B and C) Summary data of experiments (each symbol represents a different donor) showing inhibition of p-STAT5 induction in CFSE+ cells from inclusion of PL (B) or CP (C) to cells that were pulsed with IL-7. Multigroup comparison (Friedman) indicated significant differences with p = 0.002 and p = 0.0017 for phenylarsine and chlorpromazine experiments, respectively. Student t test for paired data was used to generate p values shown above.

FIGURE 4. Release of cytokine into supernatants by cells pre-exposed to IL-7. PBMCs were incubated with rIL-7 (50 ng/ml) overnight and washed twice. Cells were then replated in complete medium for 5, 30, or 120 min. At these time points the supernatants were centrifuged to remove cells and transferred to responder cells to measure p-STAT5 induction. The cells from these same cultures were washed twice and transferred to mixed cell cultures with CFSE+ responder cells. p-STAT5 expression was measured in CFSE+ cells by intracellular flow cytometry. (A) p-STAT5 induction in CD4+ CFSE+ responder cells was measured after transfer of supernatants (closed symbols and solid lines) or the transfer of cells (1:1 ratio of IL-7–pretreated cells: CFSE+ responder cells) after 15-min incubation. Statistically significant differences in p-STAT5 induction were observed between the 5-min transfer of cells and the 30- or 120-min transfer of cells (paired t test, p = 0.012 and 0.018, respectively). (B) p-STAT5 induction in transferred supernatants from the 30-min incubation time point was treated with anti–IL-7 neutralizing Ab or an isotype control Ab. Control supernatants were from cells that had been incubated overnight in medium alone and then transferred to responder cells in the same manner as the supernatants from IL-7–treated cells.
zations within lymphocytes (Fig. 7A–D). By stringent analyses of 18 independent cells, we calculated that Pearson’s $R$ values and Spearman’s rank correlation ($r$) values range from 0.45 to 0.95, indicating a relatively high degree of colocalization of the fluorescence signals from IL-7 and Rab11 (Fig. 7E). These observations provide confirmation that IL-7 can be rerouted into export pathways after internalization.

Glycosylated human IL-7 is also recycled for presentation between cells

Our studies described earlier centered on rIL-7 derived from *E. coli*. Unlike natural IL-7, this molecule is not glycosylated, raising the possibility that glycosylated IL-7 may behave differently. To test whether natural, glycosylated IL-7 can also be recycled, we pulsed PBMCs with purified IL-7 derived from HEK cells that were engineered to produce human IL-7 protein. This glycosylated IL-7 product was readily recycled in our assays such that PBMCs pulsed with cytokine and then washed were readily able to induce p-STAT5 signaling in neighboring T cells (Fig. 8). Thus, natural IL-7 also retains the capacity to be recycled for further utilization.

Discussion

Given the importance of IL-7 in T cell homeostasis, it is not surprising that cells have adapted a special mechanism to optimize IL-7 utilization. Among these mechanisms, T cells downmodulate IL-7 $\alpha$-chain expression when exposed to IL-7, resulting in reduced CD127 surface expression and less consumption of cytokine (10). In theory, this makes more cytokine available for neighboring cells and optimizes cytokine utilization. Our data...
suggest that IL-7 can be recycled, thereby permitting further “sharing” of cytokine signals.

Previous studies have suggested that bone marrow stromal cells may have a unique role in presenting IL-7 in trans to developing B cells (25). In this model, stromal cell–associated heparin sulfates are thought to mediate an interaction with IL-7 that permits binding and subsequent delivery of the cytokine to nearby cells (25). Other studies demonstrated a low-affinity interaction between heparin sulfate and IL-7 but did not find evidence that IL-7 bound directly to the surface of the S17 stromal cell line (26). Our observations suggest a novel pathway of IL-7 delivery between T lymphocytes that seems to rely on the expression of CD127 on both the cell presenting IL-7 and the cell receiving the signal. Our observations suggest a role for IL-7R internalization and subsequent recycling of cytokine to the cell surface for release as an important component of IL-7 transfer between cells. Notably, the detailed molecular mechanism that accounts for rerouting internalized IL-7 into the exocytic pathway remains to be elucidated.

Our studies largely rule out the possibility that IL-7 is simply carried over after washing cells pre-exposed to cytokine and subsequently used to deliver IL-7 to neighboring cells. If this was the case, we would expect cells placed in transwells to respond to IL-7 as a result of cytokine carryover instead of only finding responses in the responder cells that are mixed directly with cells pre-exposed to IL-7 (Fig. 1). Moreover, it is difficult to reconcile cytokine supernatant carryover with the fact that anti–IL-7R Ab and inhibitors of endocytosis affect the ability of cells to deliver IL-7 to neighboring cells (Figs. 2 and 3). Thus, it seems clear that most of the activity that we observe in these assays is due to cell-associated IL-7 and not from supernatant carryover.

Recent insights into IL-7R recycling may be related to our observations (17). CD127 is regularly recycled to the cell surface in the absence of IL-7; however, in the presence of IL-7, receptor recycling decreases as degradation increases. IL-7–induced receptor degradation has been linked to JAK3 signaling, which appears to rely on endocytosis of the receptor–cytokine complex.
We propose that this process of internalization and trafficking to early endosomes may also include a mechanism to dissociate cytokine from receptor and ultimately permit at least a fraction of internalized cytokine to be rerouted to the cell surface for release and potential reutilization.

Currently, we are uncertain about the efficiency of IL-7 recycling, which is likely to depend on availability of α-chain receptors for capture, internalization of the cytokine, endosomal trafficking of cytokine–receptor complexes for rerelease of cytokine, and proximity of T cells to each other and their source of cytokine. IL-7 recycling was detected in cells pulsed with as low as 0.1 ng/ml cytokine in vitro. This activity was measured at a 1:1 ratio of IL-7 pulsed cells to responder cells. In the context of a lymph node, however, T cells may be surrounded by many neighboring cells that could potentially recycle IL-7. Thus, the significance of IL-7 recycling in vivo remains to be determined, although our data suggest that this process is at least a feasible mechanism to optimize IL-7 utilization.

Acknowledgments
We thank Oliver Hartley (University of Geneva) for discussion of experiments.

Disclosures
The authors have no financial conflicts of interest.

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