

# TLR3 agonists improve the immunostimulatory potential of cetuximab against EGFR<sup>+</sup> head and neck cancer cells

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Toll-like receptor 3 (TLR3) agonists have been extensively used as adjuvants for anticancer vaccines. However, their immunostimulatory effects and precise mechanisms of action in the presence of antineoplastic monoclonal antibodies (mAbs) have not yet been evaluated. We investigated the effect of TLR3 agonists on cetuximab-mediated antibody-dependent cellular cytotoxicity (ADCC) against head and neck cancer (HNC) cells, as well as on dendritic cell (DC) maturation and cross-priming of epidermal growth factor receptor (EGFR)-specific CD8<sup>+</sup> T cells. The cytotoxic activity of peripheral blood mononuclear cells (PBMCs) or isolated natural killer (NK) cells expressing polymorphic variants (at codon 158) of the Fc $\gamma$  receptor IIIa (Fc $\gamma$ RIIIa) was determined in <sup>51</sup>Cr release assays upon incubation with the TLR3 agonist poly-ICLC. NK cell stimulation was measured based on activation and degranulation markers, while DC maturation in the presence of poly-ICLC was assessed using flow cytometry. The DC-mediated cross priming of EGFR-specific CD8<sup>+</sup> T cells was monitored upon in vitro stimulation with tetramer-based flow cytometry. TLR3-stimulated, unfractionated PBMCs from HNC patients mediated robust cetuximab-dependent ADCC, which was abrogated by NK-cell depletion. The cytolytic activity of TLR3-stimulated NK cells differed among cells expressing different polymorphic variants of Fc $\gamma$ RIIIa, and NK cells exposed to both poly-ICLC and cetuximab expressed higher levels of CD107a and granzyme B than their counterparts exposed to either stimulus alone. Poly-ICLC plus cetuximab also induced a robust upregulation of CD80, CD83 and CD86 on the surface of DCs, a process that was partially NK-cell dependent. Furthermore, DCs matured in these conditions exhibited improved cross-priming abilities, resulting in higher numbers of EGFR-specific CD8<sup>+</sup> T cells. These findings suggest that TLR3 agonists may provide a convenient means to improve the efficacy of mAb-based anticancer regimens.

## Introduction

Targeting the epidermal growth factor receptor (EGFR), which is overexpressed in more than 90% of head and neck cancer (HNC) patients, with monoclonal antibodies (mAbs) such as cetuximab is a well-established and clinically effective therapeutic strategy.<sup>1–3</sup> If the antitumor efficacy of this mAb solely relied upon the blockade EGFR signaling in tumor cells, abrogating the delivery of proliferative, angiogenic and metastatic signals, one would expect high response rates to cetuximab therapy among EGFR<sup>+</sup> cancer patients. However, the response rate to cetuximab among patients bearing solid tumors such as colorectal carcinoma (CRC), non-small cell lung carcinoma (NSCLC) and HNC is only 10–20%, warranting efforts to enhance its antineoplastic activity by means of combinatorial approaches.<sup>1,4–9</sup> The immunological mode of action of several antineoplastic

mAbs is progressively being recognized.<sup>10–13</sup> In this setting, HNC cells may escape recognition by the immune system by downregulating non-classical MHC Class I antigens or owing to polymorphisms in the Fc $\gamma$  receptor IIIa (Fc $\gamma$ RIIIa) expressed by natural killer (NK) cells.<sup>12,14,15</sup> Irrespective of the mechanism of resistance, enhancing the immune effects of cetuximab using clinically-relevant immune modifiers and cytokines is a logical approach to improve disease outcome.<sup>16–18</sup>

Toll-like receptors (TLRs) are expressed by both effector cells of the immune system and cancer cells.<sup>19,20</sup> These receptors belong to the large family of pattern-recognition receptors (PRRs), and their primary function is to identify so-called “pathogen-associated molecular patterns” (PAMPs).<sup>20,21</sup> The activation of TLRs stimulates innate, and subsequently adaptive, immune responses via the secretion of pro-inflammatory cytokines.<sup>20</sup> Moreover, TLR agonists can directly activate immune

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effectors and hence enhance antitumor immune responses. For instance, it has recently been demonstrated that CpG-containing oligodeoxynucleotides can directly promote the secretion of cytokines by NK cells exposed to antibody-coated tumor cells by activating TLR9.<sup>22</sup>

The administration of TLR3 agonists to cancer patients induces the secretion of pro-inflammatory cytokines such as tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), interleukin-12 (IL-12) and interferon  $\gamma$  (IFN $\gamma$ ), which in turn stimulates the recruitment of effector cells to the tumor microenvironment.<sup>23–25</sup> Since patients with advanced cancer are typically immunosuppressed, TLR3 agonists may provide a convenient means of enhancing the therapeutic effects of cetuximab and alleviating tumor resistance to mAb-based immunotherapy. Of note, TLR3 agonists have also been shown to exert direct antitumor activity and to suppress metastatic spread in HNC models.<sup>26</sup>

The TLR3 ligand polyinosinic:polycytidylic acid (polyI:C) was first produced in 1967 and demonstrated a robust ability to stimulate the production of Type I IFN.<sup>27</sup> However, polyI:C exhibited significant toxicity and the subsequent modification of the compound with carboxycellulose, resulting in the stabilized form known as poly-ICLC, alleviated these initial concerns.<sup>28,29</sup> The safety and therapeutic potential poly-ICLC have been tested in several clinical trials involving patients with urological and brain neoplasms, with encouraging results.<sup>30–33</sup> Thus, poly-ICLC might also be employed as an immune adjuvant to cetuximab-based immunotherapy in HNC patients.

The objective of this study was to evaluate the effect of poly-ICLC on cetuximab-triggered NK cell-mediated ADCC as well as on dendritic cell (DC) maturation and its impact on the induction of EGFR-specific CD8<sup>+</sup> T cells. Furthermore, we wanted to examine the activation profile of NK cells expressing polymorphic variants of Fc $\gamma$ RIIIa (at codon 158), which have been correlated with the clinical response to cetuximab-based therapy.<sup>34</sup>

## Results

**Expression of TLR3 by HNC cells and direct effects of poly-ICLC.** EGFR<sup>+</sup> HNC cell lines (UM-22B and PCI-15B) were shown to express TLR3 using quantitative RT-PCR (Fig. 1A) and flow cytometry (Fig. 1B). Next, HNC cells were incubated with poly-ICLC to assess its direct cytotoxic effects. After a stimulation period of 24 h, poly-ICLC induced low levels of apoptosis in TLR3<sup>+</sup> HNC cell lines, in a dose-dependent fashion. At a dose of 20  $\mu$ g/mL or 40  $\mu$ g/mL, the percentage of apoptotic cells (AnnexinV<sup>+</sup>) increased indeed from 7.8% (baseline) to 9% (20  $\mu$ g/mL) and 10.2% (40  $\mu$ g/mL) (Fig. 2A). We therefore selected the lower dose of poly-ICLC (20  $\mu$ g/mL) for <sup>51</sup>Cr release assays, as this poly-ICLC concentration exerted low background cytotoxicity in the absence of lymphocytes (Fig. 2B).

**Poly-ICLC enhances cetuximab-dependent NK cell-mediated ADCC, which correlates with *FCGR3A* genotype.** To determine if poly-ICLC-treated lymphocytes would exhibit increased cetuximab-dependent ADCC, unfractionated peripheral blood mononuclear cells PBMCs were incubated with 20  $\mu$ g/mL poly-ICLC for 18 h and then tested in a classical <sup>51</sup>Cr release assay

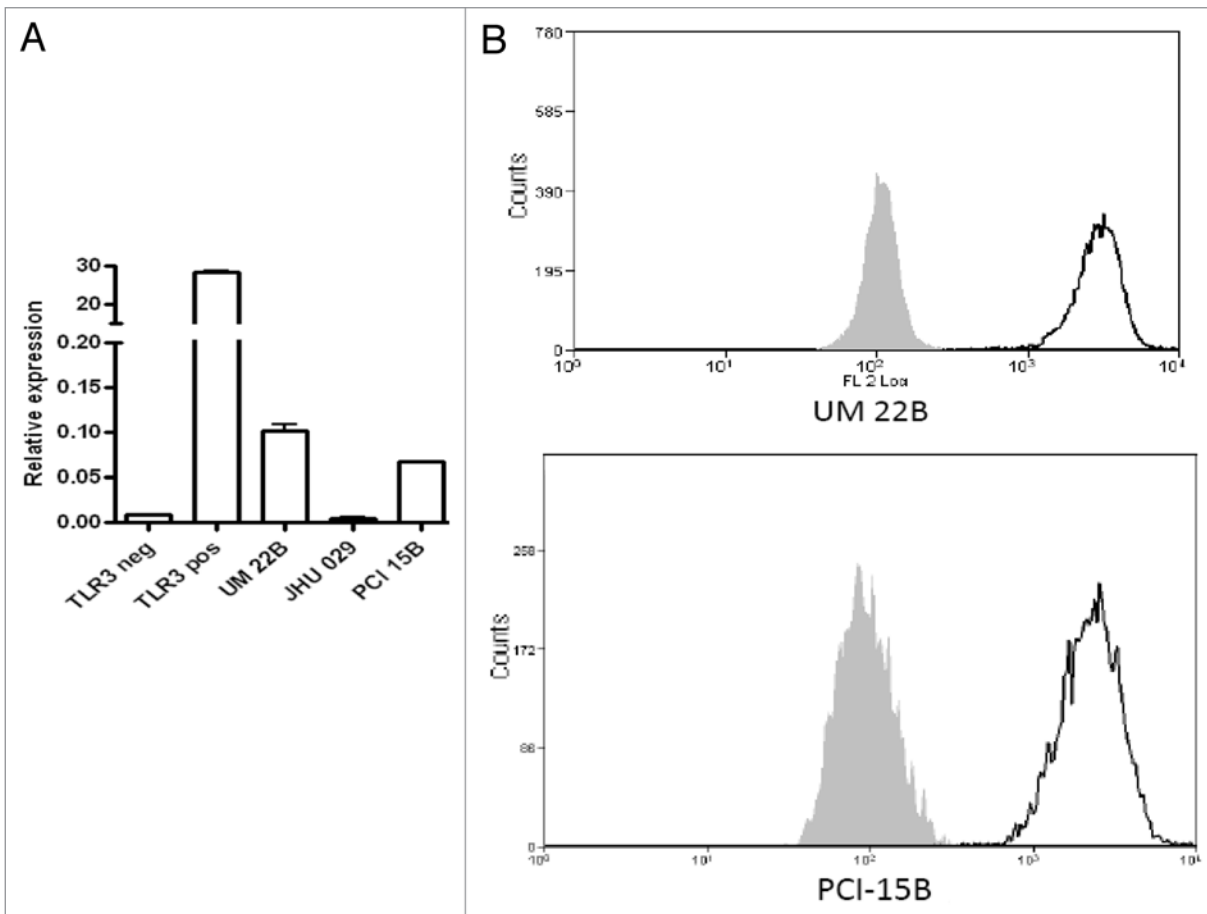
for their ability to lyse HNC cells, in the presence or absence of 10  $\mu$ g/mL cetuximab or a control IgG1. Poly-ICLC significantly enhanced cetuximab-dependent ADCC by PBMCs irrespective of Fc $\gamma$ RIIIa polymorphisms at codon 158 (F/F,  $p = 0.0081$ ; V/F,  $p < 0.0001$ ; V/V,  $p = 0.08$ ) (Fig. 3A–C). Interestingly, the PBMCs from some (approximately 25%) healthy donors failed to respond to poly-ICLC with an increase in their lytic capacity in spite of normal expression levels of TLR3 (data not shown).

When the increase in cetuximab-dependent ADCC induced by poly-ICLC was correlated with Fc $\gamma$ RIIIa polymorphic variants, PBMCs expressing Fc $\gamma$ RIIIa 158F in homozygosity were found to obtain the most consistent functional improvement from the administration of TLR3 ( $p = 0.006$ ) (Fig. 3D). We next compared the cetuximab-dependent ADCC of PBMCs expressing Fc $\gamma$ RIIIa 158F upon exposure to either 20  $\mu$ g/mL poly-ICLC or 50 IU/mL IL-2 for 18 h. Notably, poly-ICLC- and IL-2-treated PBMCs showed similar ADCC, which was significantly higher than that of vehicle-treated cells (Fig. 3E).

Poly-ICLC-receiving PBMCs from previously untreated HNC patients with active disease were found to respond to cetuximab with an increased capacity to mediate ADCC (Fig. 3F). Among these patients, 11.1% (1/9) expressed Fc $\gamma$ RIIIa 158F only, 28.6% (2/7) expressed both Fc $\gamma$ RIIIa 158F and Fc $\gamma$ RIIIa 158V and 42.9% (3/7) expressed Fc $\gamma$ RIIIa 158V only. PBMCs from two patients that did not demonstrate any lytic activity in the presence of cetuximab only became responders when poly-ICLC was included in the assays. Of these patients, one expressed both the Fc $\gamma$ RIIIa 158V and 158F variants, while the other expressed Fc $\gamma$ RIIIa 158V only, increasing the response rate to cetuximab upon poly-ICLC treatment to approximately 35%.

**NK cells mediated poly-ICLC-enhanced, cetuximab-dependent ADCC.** To determine the effector cell(s) of unfractionated PBMCs that would be responsible for the increase in cytotoxicity mediated by poly-ICLC, NK cells were depleted from PMBC preparations. NK cell-depleted PBMCs and unfractionated PBMCs (as a positive control) from the same donors were then used as effector in cetuximab-dependent ADCC assays. Poly-ICLC treated, NK-depleted PBMCs did not mediate appreciable cetuximab-dependent ADCC (Fig. 4A), demonstrating the critical contribution of NK cells to the lysis of cetuximab-coated HNC cells. To validate the importance of NK cells in the lytic activity of unfractionated PBMCs, purified NK cells were obtained by immunomagnetic separation and were used as effectors in cetuximab-dependent ADCC assays. Purified NK cells mediated indeed robust cetuximab-dependent ADCC, which was significantly enhanced in the presence of poly-ICLC (Fig. 4B). Moreover, the cytotoxicity of NK cells was abolished in the presence of a Fc $\gamma$ RIIIa-specific mAb (3G8).

**Poly-ICLC promotes NK-cell degranulation.** To demonstrate the mechanisms underlying the enhanced lytic potential of TLR3-stimulated NK cells, poly-ICLC-treated PBMCs were evaluated for the expression of degranulation markers CD107a and granzyme B using flow cytometry. CD3<sup>neg</sup>CD56<sup>+</sup>CD16<sup>+</sup> NK cells were significantly more likely to be CD107a<sup>+</sup> and granzyme B<sup>+</sup> upon exposure to poly-ICLC ( $p = 0.0019$ ) or cetuximab ( $p = 0.0009$ ) alone. In addition, the increase in activated



**Figure 1.** Expression of TLR3 in by head and neck cancer cells. **(A and B)** RNA was isolated from head and neck cancer (HNC) UM-22B, PCI-15B and JHU-029 cells as well as by TLR3-negative HEK293 cells, and the corresponding cDNAs were generated. TLR3-transfected HEK293 cells were used as a positive control. *TLR3* expression was quantified by qRT-PCR and normalized to the expression of the control gene *GUS* (coding for  $\beta$ -glucuronidase). Alternatively, TLR3 expression on UM-22B and PCI-15B cells was evaluated by flow cytometry. To this aim,  $5 \times 10^5$  cells were cultured in DMEM supplemented with L-glutamate, streptomycin and penicillin for 48 h and serum starved for the subsequent 24 h before collection. Cells were then fixed with 2% paraformaldehyde (PFA), permeabilized with 100% methanol and then labeled with an anti-TLR3 monoclonal antibody or isotype-matched antibodies and analyzed by flow cytometry. UM-22B and PCI-15B cells expressed TLR3 at both the mRNA **(A)** and protein **(B)** level.

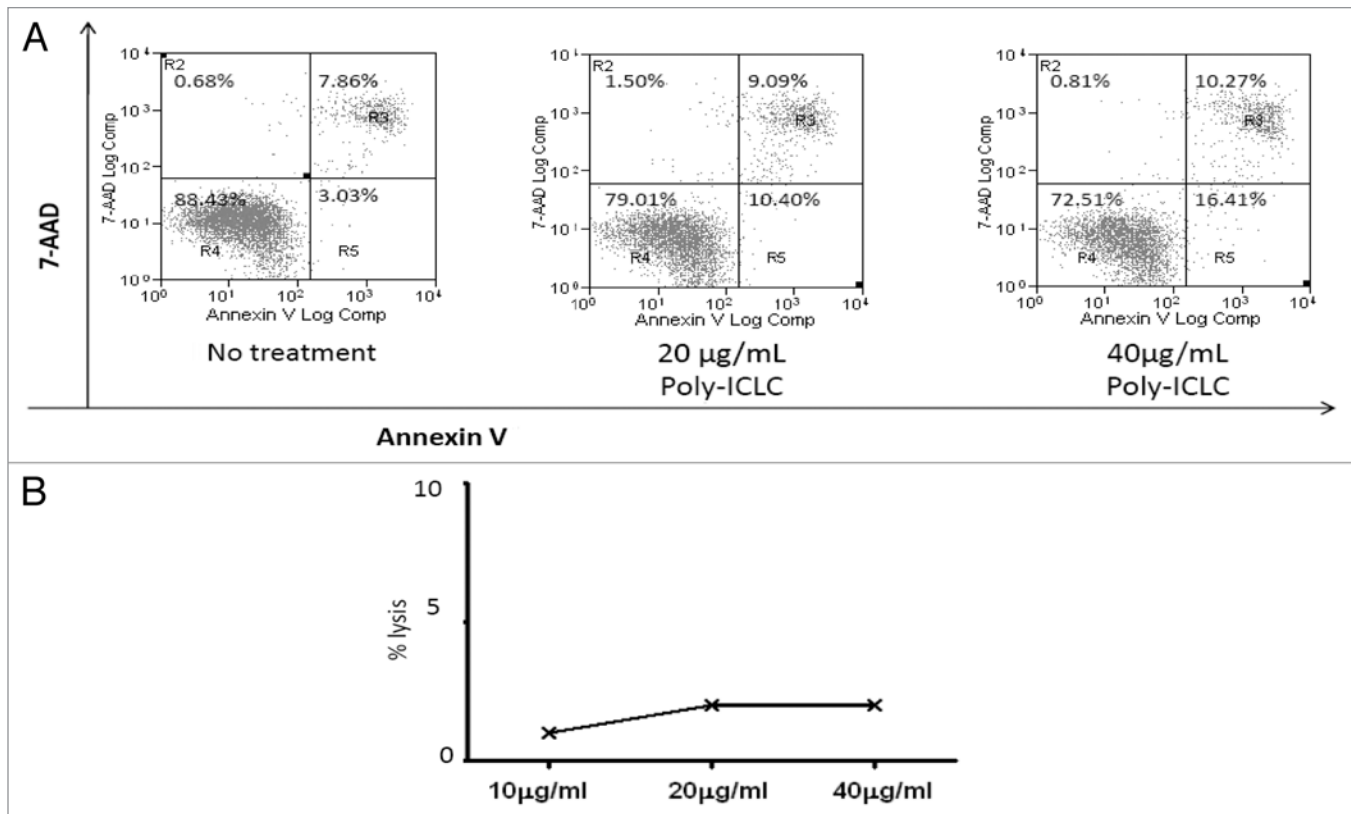
CD107a<sup>+</sup>granzyme B<sup>+</sup> NK cells was more pronounced when NK-cell cultures were treated with both poly-ICLC and cetuximab ( $p = 0.0008$ ) (Fig. 5A), consistent with their greater lytic activity against cetuximab-coated HNC cells. In addition, poly-ICLC-treated PBMCs exhibited a similar percentage of activated CD107a<sup>+</sup>granzyme B<sup>+</sup> NK cells than PBMCs exposed to IL-2. Again, the percentage of activated NK cells was dramatically increased among PBMCs treated with both cetuximab and poly-ICLC as compared with PBMCs receiving poly-ICLC or cetuximab alone (Fig. 5B).

**Poly-ICLC-stimulated NK cells enhanced DC maturation and the cross-priming of EGFR-specific CD8<sup>+</sup> T cells.** To determine whether poly-ICLC facilitated the priming of EGFR-specific CD8<sup>+</sup> T cells in vitro, we first ascertained the effects of poly-ICLC on DC maturation. We have previously described a protocol for generating monocyte-derived DCs (mDCs).<sup>11</sup> These DCs are known to express TLR3, a point that we verified by staining mDCs with a TLR3-specific antibody and flow cytometry (data not shown). Next, we incubated mDCs with 50  $\mu$ g/

mL poly-ICLC for 48 h and evaluated the upregulation of the DC maturation markers CD80, CD83 and CD86. As shown in **Figure 6A**, the stimulation of mDCs with poly-ICLC resulted in a maturation phenotype characterized by increased expression levels of CD80 ( $p = 0.03$ ), CD83 ( $p = 0.04$ ) and CD86 ( $p = 0.004$ ).

Next, we co-cultured mDCs with NK cells and PCI-15B HNC cells, alone or in the presence of 20  $\mu$ g/mL poly-ICLC, 10  $\mu$ g/mL cetuximab or both. The combination of cetuximab and poly-ICLC induced a significantly higher proportion of DCs expressing high levels of CD80, CD83 and CD86 than cetuximab or poly-ICLC alone ( $p = 0.001$ ;  $p = 0.0017$ ;  $p = 0.002$ ) (Fig. 6B).

The functional significance of DC maturation as induced by poly-ICLC and cetuximab-activated NK cells was assessed by the in vitro stimulation (IVS) of HLA-A\*0201 CD8<sup>+</sup> T cells. After a 7-d IVS period, there was nearly a 2-fold increase in the frequency of EGFR<sub>853-861</sub>-specific CD3<sup>+</sup>CD8<sup>+</sup> T cells in the co-cultures exposed to poly-ICLC and cetuximab in combination, as compared with those receiving either drug alone ( $p = 0.02$ ) (Fig. 6C). Notably, a dramatic (nearly 4-fold) enhancement in the



**Figure 2.** Poly-ICLC exerts a slight pro-apoptotic effect on UM-22B cells but no over toxicity in the absence of PBMC. **(A)** UM-22B cells were incubated with increasing concentrations of poly-ICLC for 24 h, harvested and then stained with AnnexinV and 7-AAD for flow cytometry. Poly-ICLC slightly increases the percentage of apoptotic cells (shown in bold) in a dose-dependent manner. **(B)** Alternatively, HNC cells were labeled with 1 mCi  $^{51}\text{Cr}$ , washed twice with media and incubated with the indicated concentrations of cetuximab. Finally, supernatants were harvested and radioactivity was quantified on a scintillation counter. Negligible cell lysis was documented when UM-22B cells were incubated with poly-ICLC in the absence of immune effectors.

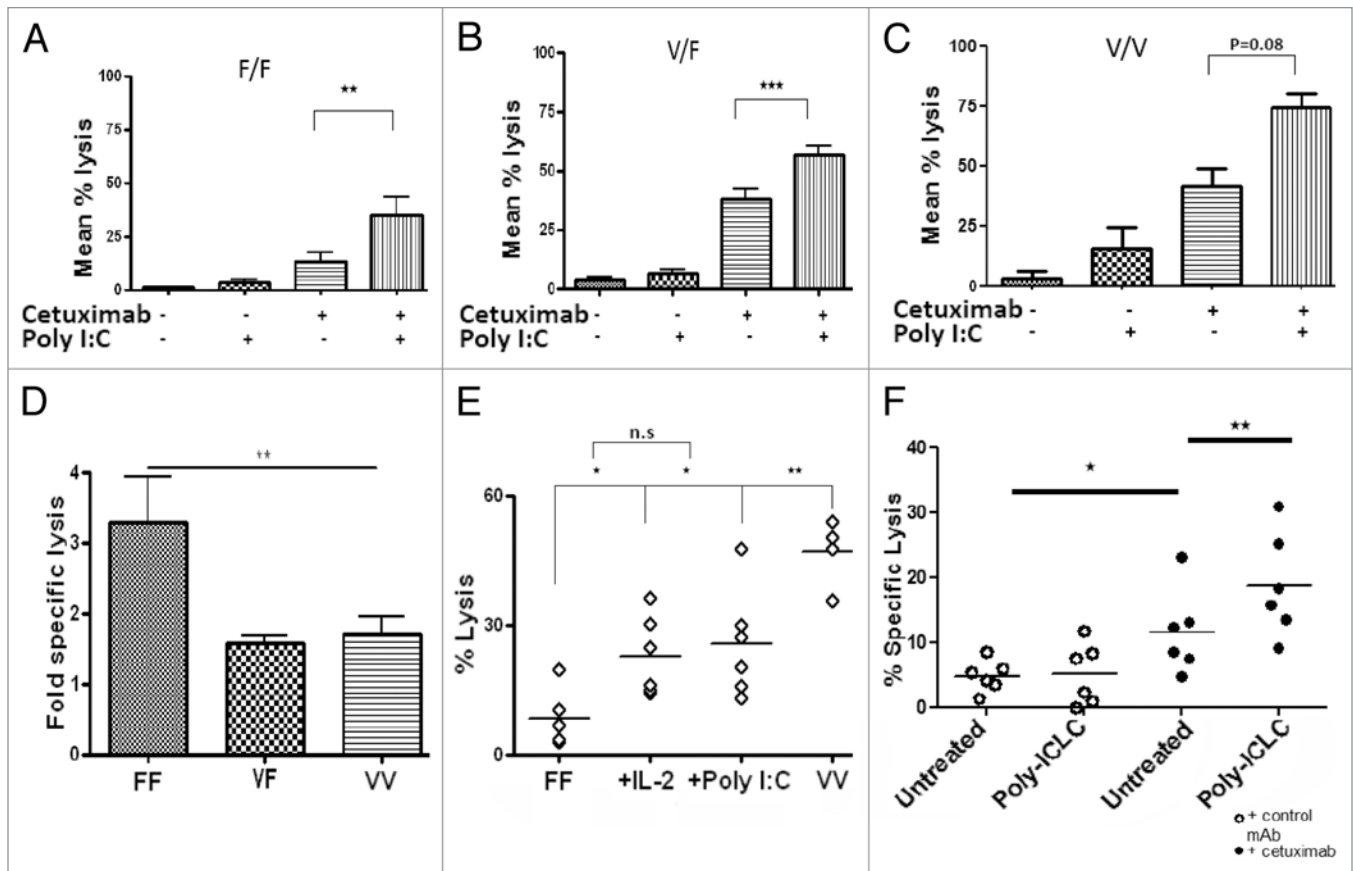
propagation of EGFR<sub>853-861</sub>-specific CD3<sup>+</sup>CD8<sup>+</sup> T cells was seen in co-cultures treated with poly-ICLC and cetuximab following a second, 7 d-long IVS ( $p < 0.0001$ ) (Fig. 6C). Therefore, the functional cross-priming activity of DCs was well pronounced in the presence of poly-ICLC and cetuximab-activated NK cells and increased significantly upon repeated IVS.

## Discussion

Enhancing host immune responses should be considered as a measure to increase the efficacy of cetuximab-based therapy in HNC patients. With the discovery that TLR agonists function as potent immune adjuvants, promoting innate immunity and the subsequent induction of adaptive immune response has become an attractive alternative in this sense.<sup>35</sup> Double stranded RNA is a natural ligand for TLR3 and drives a signaling cascade that results in the production of T<sub>H</sub>1 cytokines, which are beneficial in the tumor microenvironment.<sup>25,36,37</sup> Poly-ICLC, a TLR3 agonist, has been investigated in clinical trials for its ability to boost vaccine-induced immune responses against brain tumors, exhibiting an acceptable toxicological profile.<sup>30,33</sup> The role of TLR3 signals in the context of mAb-based therapy, however, has not yet been tested, driving us to launch this preclinical study to evaluate the

effects of poly-ICLC on cetuximab-dependent ADCC against HNC cells. The enhancement of cetuximab-dependent ADCC by poly-ICLC is significant because ADCC marks the first step of activation of the immune system during mAb-based immunotherapy. In our model, subsequent steps of activation are promoted by poly-ICLC, owing to its maturing effect on DCs, which eventually results in the activation and recruitment of EGFR specific CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs). This strategy might improve disease course among cetuximab-treated HNC patients, since the presence of a strong tumor-specific CTL response has been associated with improved clinical outcomes.<sup>38</sup>

Our findings suggest for the first time that the use of a TLR3 agonists (poly-ICLC) as an immune adjuvant constitutes a valid means to enhance the antitumor effects of cetuximab against HNC. The expression of TLR3 by immune cells and HNC cell lines was investigated as the TLR3 pathway may per se mediate pro-apoptotic effects in this context.<sup>26</sup> Previous studies have reported the intracellular expression of TLR3 by immune cells like DCs, monocytes and NK cells, and these findings were confirmed by our observations.<sup>39-41</sup> Similarly, we demonstrated that the EGFR<sup>+</sup> HNC cell lines used in our experiments (UM-22B and PCI-15B) express TLR3. This information is clinically relevant as agonists of other TLRs such as TLR4 have been suggested



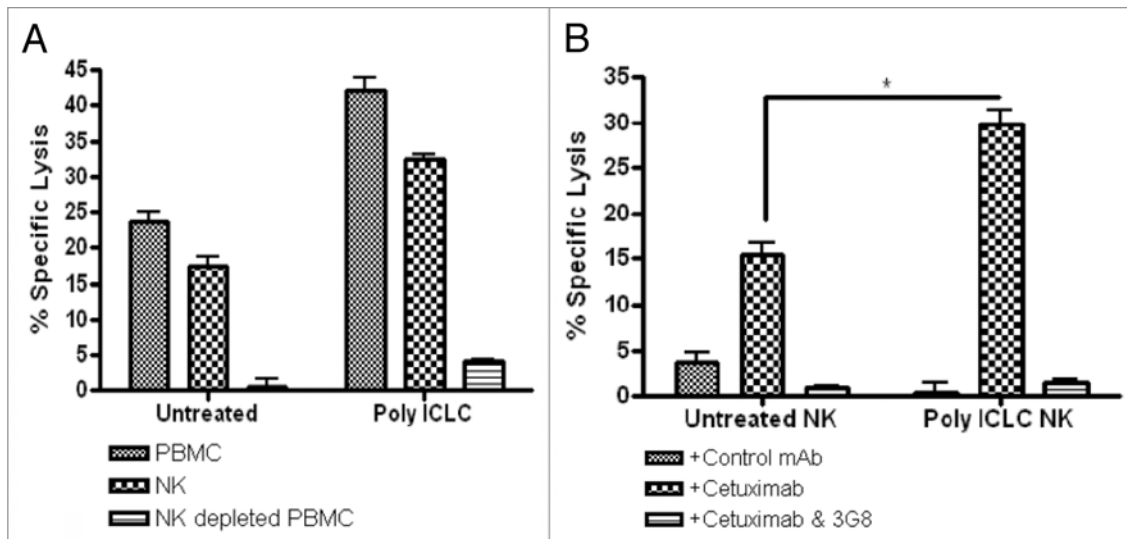
**Figure 3.** Poly-ICLC enhances cetuximab-dependent ADCC by peripheral blood mononuclear cells from healthy donors. (A–E) UM-22B or PCI-15B HNC cells were used as targets in  $^{51}\text{Cr}$  release antibody-dependent cellular cytotoxicity (ADCC) assays, using natural killer (NK) cells expressing Fc $\gamma$ R1IIa 158F only (F/F), both Fc $\gamma$ R1IIa 158F and 158V (V/F) or Fc $\gamma$ R1IIa 158V only (V/V). F/F NK cells demonstrated the greatest increase in ADCC upon TLR3 stimulation ( $p = 0.006$ ). Healthy donor-derived peripheral blood mononuclear cells (PBMCs) were left untreated or treated with 50  $\mu\text{g}/\text{mL}$  poly-ICLC for 18 h and then co-cultured for 4 h with  $^{51}\text{Cr}$ -labeled UM-22B or PCI-15B cells in the presence of 10  $\mu\text{g}/\text{mL}$  cetuximab or isotype-matched IgG1 at an E:T ratio of 50:1. IL-2-treated PBMCs were used as a positive control. Genomic DNA extracted from PBMCs was used to determine the genotype at the Fc $\gamma$ R1IIa-coding locus by PCR. (A–C) Effects of poly-ICLC on cetuximab-dependent ADCC using F/F (A), V/F (B) or V/V (C) PBMCs. (D) F/F NK cells derived the greatest functional benefits (approximately 3-fold increase) from poly-ICLC as compared with a 1-fold increase among V/F and V/V cells. (E) The ADCC activity of poly-ICLC-treated F/F PBMCs was significantly enhanced by poly-ICLC, comparing favorably with that of IL-2-treated PBMCs. (F) PBMC harvested from HNC patients were treated with 50  $\mu\text{g}/\text{mL}$  poly-ICLC for 18 h prior to incubation with cetuximab-coated,  $^{51}\text{Cr}$ -labeled UM-22B or PCI-15B cells at an E:T ratio of 50:1, for 4 h. Supernatants were then harvested and radioactivity was quantified on a scintillation counter. Poly-ICLC enhanced cetuximab-dependent ADCC in PBMCs from 6 out of 23 (26.1%) HNC patients, 11.1% of which were F/F PBMCs, 28.6% V/F and 42.9% V/V PBMCs ( $p = 0.0028$ ).

to protect HNC cells from immune attacks.<sup>22</sup> In line with results from previous studies,<sup>20,26</sup> TLR3 ligation did not mediate any pro-survival effect in HNC cells, further supporting our intent to study the immunostimulatory effects of poly-ICLC on cetuximab-based immunotherapy.<sup>18</sup> On the contrary, we documented a modest, dose-dependent pro-apoptotic effect of poly-ICLC on HNC cells.

We had previously established the impact of Fc $\gamma$ R1IIa polymorphisms on cetuximab-dependent ADCC against HNC, with the highest lytic effect seen for NK cells expressing Fc $\gamma$ R1IIa 158V only, followed by cells expressing both Fc $\gamma$ R1IIa 158V and 158F and cells expressing Fc $\gamma$ R1IIa 158F only.<sup>14</sup> Using  $^{51}\text{Cr}$  release assays to determine the effect of poly-ICLC on cetuximab-dependent ADCC, we demonstrated that poly-ICLC stimulates ADCC independent of Fc $\gamma$ R1IIa polymorphisms. Such an increase concerned immune cells, as it was observed only in the presence of

PBMCs. No measurable cytotoxicity indeed developed when PBMCs were not added to ADCC assays. Furthermore, we demonstrated that the ADCC-stimulating effects of poly-ICLC are primarily mediated by NK cells, as they were abrogated when NK-depleted PBMCs were used as effectors in ADCC assays. NK cells mediated cetuximab-dependent ADCC via the CD16 receptor, as no measurable lysis was seen when an anti-CD16 mAb (3G8) was added to the assays.

To characterize the functional activation of NK cells by poly-ICLC, we employed flow cytometry to evaluate the upregulation of NK-cell degranulation markers (CD107a and granzyme B) in the course of cetuximab-dependent ADCC. The percentage of activated NK cells characterized by a CD107a<sup>+</sup>granzymeB<sup>+</sup> phenotype was modestly increased by poly-ICLC or cetuximab alone, yet augmented to nearly 4-fold in the presence of both agents. Furthermore, the functional activation of NK cells induced by



**Figure 4.** NK cells are the primary effectors of poly-ICLC-stimulated cetuximab-dependent ADCC. **(A and B)** NK cells were purified from peripheral blood mononuclear cells (PBMCs) harvested from healthy donors using magnetic bead separation. Purified NK cells were treated with 50  $\mu$ g/mL poly-ICLC for 18 h and then used as effectors in  $^{51}$ Cr release assays involving cetuximab-coated UM-22B or PCI-15B cells (E:T ratio = 10:1). Similarly, NK-depleted PBMCs were treated with poly-ICLC and cetuximab-dependent ADCC was monitored in  $^{51}$ Cr release assay (E:T ratio = 10:1). **(A)** Poly-ICLC failed to enhance cetuximab-dependent ADCC when the NK-depleted PDMC fraction was used as effector. **(B)** Poly-ICLC enhanced cetuximab-dependent ADCC when purified NK cells were used as effectors. The lytic activity of PBMCs and NK cells was completely abrogated when an anti-CD16 antibody (3G8) was added to effector cell/target cell co-cultures.

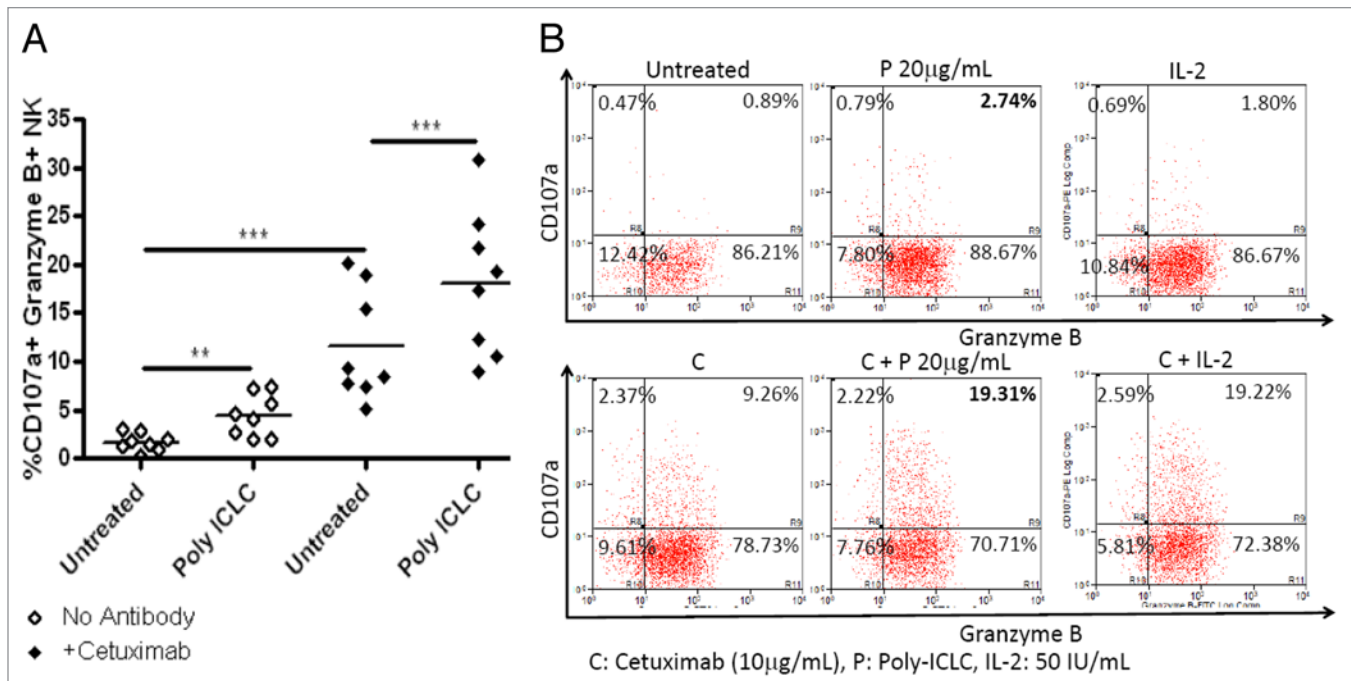
poly-ICLC and IL-2 was similar. While others have analyzed the use of cytokines, such as IL-2, IL-12 and IL-21, to enhance the therapeutic efficacy of mAbs, the clinical utility of this approach is limited by the toxicity of systemic cytokine administration. Agonists of other TLRs, such as TLR7, TLR8 and TLR9, have also been investigated in ongoing or published studies for their immunostimulatory properties.

When cetuximab-dependent ADCC was evaluated on PBMCs obtained from HNC patients, the overall response rate was 26.1%. This rate mirrored the response rate to cetuximab seen in clinical trials involving HNC patients (20–30%), suggesting a correlation between immune responses to cetuximab-based therapy and disease course.<sup>1,3,42</sup> Poly-ICLC further enhanced the cytotoxic functions mediated by PMBCs isolated from cetuximab responders. Furthermore, PBMCs (from two distinct HNC patients) that did not demonstrate lytic activity in the presence of cetuximab only became able to mediate ADCC when incubated with poly-ICLC.

The response rate of NK cells from HNC patients expressing Fc $\gamma$ RIIIa 158F only to cetuximab was 11.1%, and poly-ICLC was unable to restore cetuximab-dependent ADCC among the non-responders of this group. Overall, the mean percentage of ADCC responses of patient-derived NK cells expressing Fc $\gamma$ RIIIa 158F only was 3.9%. Given the extremely low ADCC response rate of NK cells from these HNC patients, we postulate that combination of cetuximab with poly-ICLC might be particularly beneficial in this setting. Enhancing the cetuximab-dependent cytotoxicity of NK cells from HNC patients expressing both Fc $\gamma$ RIIIa 158V and 158F or Fc $\gamma$ RIIIa 158V only by means of with poly-ICLC was also feasible, since the mean percentage of ADCC responses in this setting was 10.7%.

This hypothesis is in accordance with our immunological model of mAb-based immunotherapy for HNC.<sup>3</sup> Since ADCC is an important mechanism used by innate effectors such as NK cells to elicit a first wave of immunogenic death among cancer cells, strategies aimed at enhancing the ADCC response of HNC patients are likely to result in improved adaptive immune responses and therapeutic outcome. To provide evidence in support of the activation of the adaptive immune system by TLR3 agonists combined with cetuximab-mediated immunotherapy, we demonstrated that poly-ICLC and cetuximab induce a robust upregulation of DC maturation markers such as CD80, CD83 and CD86. The maturation of DCs induced by poly-ICLC plus cetuximab had functional downstream consequences, as demonstrated by a synergistic increase in EGFR-specific CD8<sup>+</sup> T cells secondary to enhanced DC-mediated cross-priming. Our findings are clinically relevant as they validate the immunological mechanism of action of cetuximab-based immunotherapy against HNC and demonstrate that TLR3 agonists can be used as immune adjuvants to enhance both innate and adaptive HNC-specific immune responses elicited by cetuximab.

In summary, the activation of TLR3 signaling with poly-ICLC exerts a beneficial effect on NK cells, resulting in the increased cetuximab-dependent lysis of HNC cells. Poly-ICLC also enhances DC maturation and the cross-priming of tumor-specific CD8<sup>+</sup> cells. Finally, there is a modest, dose-dependent pro-apoptotic effect of poly-ICLC on TLR3<sup>+</sup> HNC cell lines. Overall, our study demonstrates for the first time that TLR3 agonists such as poly-ICLC can be employed as immune adjuvants to enhance cetuximab-dependent ADCC against HNC and the consequent elicitation of adaptive antitumor immune responses.



**Figure 5.** Poly-ICLC plus cetuximab increase the percentage of NK cells characterized by a CD107a<sup>+</sup>granzymeB<sup>+</sup> phenotype. **(A and B)** Peripheral blood mononuclear cells (PBMCs) harvested from healthy donors were left untreated or treated with 50 µg/mL poly-ICLC for 18 h. Thereafter, PBMCs were co-cultured with PCI-15B cells for 4 h at a 1:1 ratio in the absence or in the presence of 10 µg/mL cetuximab. NK cells were identified upon immunostaining as CD3<sup>neg</sup>CD56<sup>+</sup>CD16<sup>+</sup> cells, and the percentage of CD107a<sup>+</sup>granzymeB<sup>+</sup> NK cells were assessed by flow cytometry. **(A)** Poly-ICLC and cetuximab alone increase the fraction of CD107a<sup>+</sup>granzymeB<sup>+</sup> NK cells ( $p = 0.0019$  and  $p = 0.0009$ , respectively), an effect that is further increased in the presence of both poly-ICLC and cetuximab ( $p = 0.0008$ ). **(B)** Poly-ICLC plus cetuximab increased the fraction of CD107a<sup>+</sup>granzymeB<sup>+</sup> NK cells more significantly than poly-ICLC alone, comparing favorably with 50 IU/mL IL-2 (employed as a positive control).

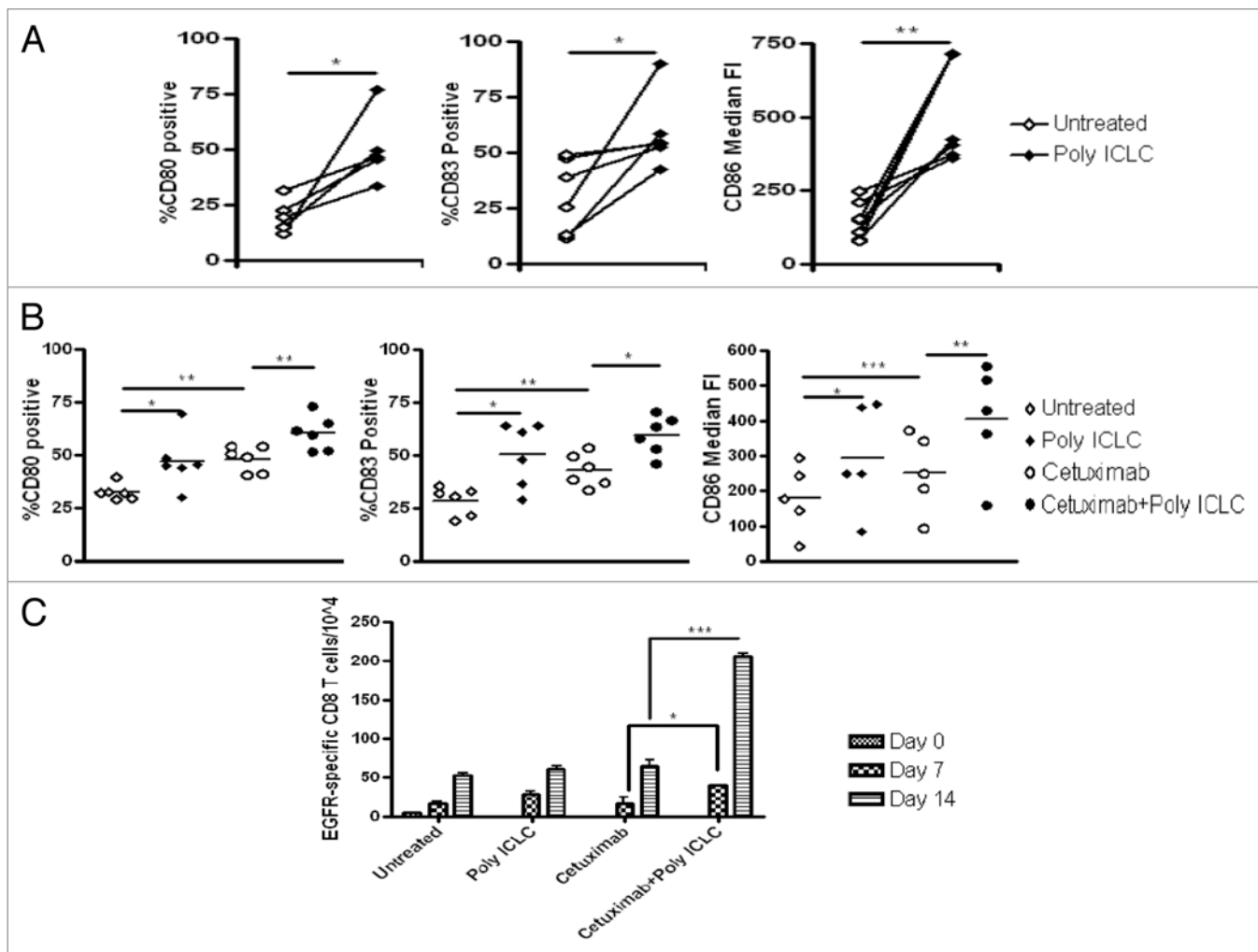
## Materials and Methods

**Cell culture.** Two EGFR<sup>+</sup> HNC cell lines (UM-22B and PCI-15B) were cultured at 37°C and under 5% CO<sub>2</sub> in Dulbecco's modified Eagle's Medium (DMEM, from Gibco-BRL, Life Technologies, Inc.) supplemented with 5% streptomycin, 2% L-glutamine (Gibco) and 10% fetal bovine serum (FBS, from Hyclone). Blood samples from healthy donors were purchased from the Western Pennsylvania blood bank and blood samples from untreated HNC patients were obtained in accordance to the University of Pittsburgh Medical Center Institutional Review Board approval protocol. PBMCs were extracted using a Ficoll-Hypaque gradient (Amersham Biosciences). Highly purified NK cells were obtained from PBMCs using the EasySep NK negative isolation kit (StemCell Technologies, Inc.), according to the manufacturer's protocol. An NK purity > 95% was invariably confirmed by flow cytometry.

**Antibodies and flow cytometry.** Cetuximab (Erbix<sup>TM</sup>, Bristol Myers Squibb) was purchased from the Hillman Cancer Center pharmacy. A human IgG1 isotype control was purchased from Enzo Life Science (ALX-804-133). Poly-ICLC (Hiltonol) was supplied by Oncovir Inc. as a courtesy of Dr. Hideho Okada (University of Pittsburgh). PE-conjugated TLR3 mAbs (9039) and PE-conjugated isotype IgG1s (4714) were purchased from eBioscience, while the CD16-specific mAb 3G8 (555404) was obtained from BD Biosciences. The following

fluorophore-conjugated antibodies/molecules were used immunostaining prior to flow cytometry: CD3-Alexa 405 (0326), purchased from Life Technologies Inc.; CD16-PE-Cy7 (302015), Granzyme B-FITC (515403), EpCAM-APC (324207), CD11c-PE-Cy7 (301607) and CD86-PE (305405), purchased from Biologend; CD56-APC (555518), CD8-APC (555369), CD80-FITC (557226), CD83-PE (556855), CD107a-PE (555801), HLA-A\*0201-FITC (551285) and 7-AAD (559925) all purchased from BD Pharmingen. Unfractionated PBMCs ( $1 \times 10^6$  cells) or purified NK cells ( $5 \times 10^5$  cells) were treated with 20 µg/mL poly-ICLC for 18 h. Thereafter, cells were incubated with CD56-, CD16- and CD3-specific labeled mAbs in 2% bovine serum albumin (BSA, w:v in PBS) for 30 min at 4°C. Following two washes in culture medium, cells were fixed with 2% paraformaldehyde (PFA) and analyzed with a Beckman Coulter EPICS XL flow cytometer. Data were analyzed using the Summit 4.3 software (Beckman Coulter).

**NK-cell degranulation.** For NK-cell degranulation experiments, PBMCs were treated with 20 µg/mL poly-ICLC or cultured in RPMI 1640 medium (Gibco-BRL, Life Technologies, Inc.) without stimulation for 18 h. PBMCs were then co-cultured with PCI-15B cells at 1:1 ratio in the absence or in the presence of 10 µg/mL cetuximab plus brefeldin A (555029, BD Pharmingen) and anti-CD107a or isotype-matched control antibodies for 4 h. PBMCs were then harvested, stained with anti-CD3, CD56 and CD16 mAbs, permeabilized using the BD Cytofix/Cytoperm



**Figure 6.** Poly-ICLC-treated dendritic cells enhance the cetuximab-dependent induction of EGFR-specific CD8<sup>+</sup> T-cells in the presence of NK cells and PCI-15B cells. **(A)** HLA-A\*0201<sup>+</sup> dendritic cells (DCs) were treated with 50  $\mu$ g/mL poly-ICLC for 48 h. The maturation of DCs by poly-ICLC resulted in the upregulation of CD80 ( $p = 0.03$ ), CD83 ( $p = 0.04$ ) and CD86 ( $p = 0.004$ ). Alternatively, HLA-A\*0201<sup>+</sup> DCs were incubated with natural killer (NK) cells and PCI-15B head and neck cancer (HNC) cells at a 1:1:1 ratio in the presence of medium only, 50  $\mu$ g/mL poly-ICLC, 10  $\mu$ g/mL cetuximab or both. **(B)** Poly-ICLC plus cetuximab induced a significantly higher proportion of DCs expressing CD80, CD83 and CD86 than 10  $\mu$ g/mL cetuximab or 50  $\mu$ g/mL poly-ICLC alone ( $p = 0.001$ ;  $p = 0.0017$ ;  $p = 0.002$ ). An in vitro stimulation assay was performed using autologous CD8<sup>+</sup> T cells incubated with DCs treated as described above for 7 or 7+7 d. **(C)** The functional cross-priming effects of DCs to induce EGFR-specific CD8<sup>+</sup> T cells following 7-d stimulation were more pronounced when DCs were matured with poly-ICLC plus cetuximab ( $p = 0.02$ ). A synergistic (nearly 4-fold) increase in the accumulation of EGFR-specific CD8<sup>+</sup> T cells was seen in co-cultures treated with poly-ICLC plus cetuximab following a second 7-d-long stimulation period ( $p < 0.0001$ ).

reagent (BD Pharmingen) and then stained for intracellular granzyme B expression. At analysis, doublet-excluding events were gated for CD3<sup>neg</sup>CD56<sup>+</sup>CD16<sup>+</sup>granzyme B<sup>+</sup> lymphocytes and analyzed for the percentage CD107a<sup>+</sup> cells.

**<sup>51</sup>Cr release assays.** PBMCs were pretreated with 20  $\mu$ g/mL poly-ICLC for 18 h. Next,  $1 \times 10^6$  HNC cells were labeled with 1 mCi Na<sup>51</sup>CrO<sub>4</sub> for 1 h at 37°C. Cells were washed twice with medium and incubated with effector cells at an E:T ratio of 50:1 in the presence of 10  $\mu$ g/mL cetuximab. Four hours later, supernatants were harvested and counted on a Microbeta Trilux scintillation counter (PerkinElmer). Percentage cytotoxicity was calculated using the formula (experimental-spontaneous release)/(maximum-spontaneous release)  $\times$  100%; where spontaneous = release from targets incubated with medium alone

and maximum = release from targets incubated by 5% Triton X-100 (Sigma Aldrich).

**Apoptosis assay.** HNC cells ( $2.5 \times 10^5$ ) were cultured in complete DMEM in T25 flasks for 2 d. Cells were observed under microscopy to ensure adequate growth and no spontaneous cell death and then treated with varying concentrations of poly-ICLC for 24 h. Cells were then harvested and stained with AnnexinV and 7-AAD (BD Pharmingen), according to manufacturer's instructions. Cytofluorometric acquisition was performed on a Beckman Coulter EPICS XL flow cytometer.

**DC culture.** After typing for HLA-A\*0201, monocytes were isolated from PBMCs by plastic adherence for 2 h at 37°C. Non-adherent cells were removed and adherent cells were incubated with Aim-V medium (Invitrogen) supplemented with



1000 IU/mL granulocyte macrophage colony-stimulating factor (GM-CSF, from R&D Systems) 1000 IU/mL IL-4 (from R&D Systems). Three days later, GM-CSF and IL-4 were replenished to the final concentration of 1000 IU/mL. mDCs were harvested on day 6 using 0.25% trypsin-EDTA (25200-072, Life Technologies, Inc.) and co-cultured with NK and PCI-15B cells (ratio 1:1) for 48 h. During this time, co-cultures were left untreated or received 50 µg/mL poly-ICLC, 10 µg/mL cetuximab or both. Cells were then harvested and stained with CD80-FITC plus CD86-PE or CD83-PE antibodies and analyzed on flow cytometry. Doublet-excluding events were gated based on high side scatter and EpCAM<sup>neg</sup>CD11c<sup>+</sup> phenotype and analyzed for the percentage of CD80<sup>+</sup> and CD83<sup>+</sup> cells as well as for and CD86 mean fluorescence intensity (MFI).

**In vitro CD8<sup>+</sup> T-cell stimulation.** Autologous HLA-A\*0201 CD8<sup>+</sup> T cells were harvested using the EasySep human T-cell enrichment kit (StemCell Technologies, Inc.), according to the manufacturer's instructions. CD8<sup>+</sup> T cells were then stimulated in vitro with DCs matured as described above either once for 7 d, or twice (14 d in total) in the presence of 20 IU/mL IL-2 (R&D Systems). Wild-type EGFR<sub>853-861</sub> was produced by the peptide synthesis facility at the University of Pittsburgh, using the F-moc technology.<sup>11,43</sup> The corresponding EGFR<sub>853-861</sub> tetramer was synthesized by the NIH Tetramer Facility (Emory University).

At the end of the stimulation, CD8<sup>+</sup> T cells were harvested and stained with CD3- and CD8-specific antibodies, 7-AAD and the EGFR<sub>853-861</sub> tetramer and analyzed by flow cytometry. Doublet-excluding events were gated for viable (7-AAD<sup>neg</sup>) lymphocytes exhibiting a CD3<sup>+</sup>CD8<sup>+</sup> phenotype and analyzed for EGFR<sub>853-861</sub> tetramer<sup>+</sup>/10,000 CD8<sup>+</sup> T cells.

**Statistical analyses.** Significant differences in ADCC by FcγRIIIa phenotype were determined using a Kruskal-Wallis one-way analysis of variance with p values < 0.05 considered as statistically significant. A post-hoc Mann-Whitney non-parametric t-test was performed for differences between groups, with p values < 0.05 considered as statistically significant.

#### Disclosure of Potential Conflicts of Interest

Dr. Andres M. Salazar is CEO and scientific director of Oncovir, Inc. The other authors do not have any conflict of interest to disclose.

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