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Overcoming regulatory T-cell suppression by a lyophilized preparation of *Streptococcus pyogenes*

Michiko Hirayama[*1,2], *Hiroyoshi Nishikawa*[*1,3], *Yasuhiro Nagata*[4], *Takemasa Tsuji*[5], *Takuma Kato*[6], *Shinichi Kageyama*[2], *Shugo Ueda*[7], *Daisuke Sugiyama*[3], *Sahoko Hori*[1,2], *Shimon Sakaguchi*[3], *Gerd Ritter*[5], *Lloyd J. Old*[5], *Sacha Gnjatic*[5] *and Hiroshi Shiku*[1,2]

[1] Department of Cancer Vaccine, Mie University Graduate School of Medicine, Mie, Japan
[2] Department of Immuno-Gene Therapy, Mie University Graduate School of Medicine, Mie, Japan
[3] Experimental Immunology, Immunology Frontier Research Center, Osaka University, Osaka, Japan
[4] Department of Surgery, National Hospital Organization Nagasaki Medical Center, Nagasaki, Japan
[5] Ludwig Institute for Cancer Research, New York Branch, Memorial Sloan-Kettering Cancer Center, New York, NY, USA
[6] Department of Cellular and Molecular Immunology, Mie University Graduate School of Medicine, Mie, Japan
[7] Department of Gastroenterological Surgery and Oncology, Kitano Hospital, Tazuke Kofukai Medical Research Institute, Osaka, Japan

Cancer vaccines have yet to yield clinical benefit, despite the measurable induction of humoral and cellular immune responses. As immunosuppression by $CD4^+CD25^+$ regulatory T (Treg) cells has been linked to the failure of cancer immunotherapy, blocking suppression is therefore critical for successful clinical strategies. Here, we addressed whether a lyophilized preparation of *Streptococcus pyogenes* (OK-432), which stimulates Toll-like receptors, could overcome Treg-cell suppression of $CD4^+$ T-cell responses in vitro and in vivo. OK-432 significantly enhanced in vitro proliferation of $CD4^+$ effector T cells by blocking Treg-cell suppression and this blocking effect depended on IL-12 derived from antigen-presenting cells. Direct administration of OK-432 into tumor-associated exudate fluids resulted in a reduction of the frequency and suppressive function of $CD4^+CD25^+Foxp3^+$ Treg cells. Furthermore, when OK-432 was used as an adjuvant of vaccination with HER2 and NY-ESO-1 for esophageal cancer patients, NY-ESO-1–specific $CD4^+$ T-cell precursors were activated, and NY-ESO-1–specific $CD4^+$ T cells were detected within the effector/memory T-cell population. $CD4^+$ T-cell clones from these patients had high-affinity TCRs and recognized naturally processed NY-ESO-1 protein presented by dendritic cells. OK-432 therefore inhibits Treg-cell function and contributes to the activation of high-avidity tumor antigen-specific naive T-cell precursors.

Keywords: Cancer · Treg cells · Tumor immunology · Vaccination

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Correspondence: Dr. Hiroyoshi Nishikawa
e-mail: nisihiro@ifrec.osaka-u.ac.jp

*These authors contributed equally to this work.

Introduction

Many tumor-associated antigens recognized by the immune system are normal self-constituents, and tumor immunity is considered to be in part an autoimmune response [1–3]. Therefore, mechanisms for maintaining immunological self-tolerance hamper effective anticancer immunity. $CD4^+CD25^+$ Treg cells are one of the major components in maintaining immunological self-tolerance in hosts by suppressing a wide range of immune responses [4–7]. Indeed, depletion of Treg-cell populations enhances spontaneous and vaccine-induced antitumor immune responses [6, 8, 9], and the stimulation of $CD4^+CD25^+$ Treg cells by immunization with self-antigens induces enhanced chemically induced primary tumor development and increased numbers of pulmonary metastasis following injection of transplantable tumor cells [10–12]. In human cancers, the presence of high numbers of $CD4^+CD25^+$ Treg cells or low ratio of $CD8^+$ T cells to $CD4^+CD25^+$ Treg cells in tumors is correlated with unfavorable prognosis [13, 14]. In addition, the depletion of $CD4^+CD25^+$ Treg cells in patients receiving a DC vaccine enhances the stimulation of tumor-specific T-cell responses, indicating a crucial role for Treg cells in the regulation of antitumor immune responses in humans [15].

NY-ESO-1, a germ cell protein, was found by SEREX (serological identification of antigens by recombinant expression cloning) using the serum of an esophageal cancer patient [16, 17]. We have previously shown that NY-ESO-1–specific $CD4^+$ T cells are detectable in cancer patients with spontaneous NY-ESO-1 serum Ab responses [17, 18]. In addition, NY-ESO-1–specific $CD4^+$ T-cell precursors can expand and become detectable in healthy individuals after in vitro antigenic stimulation of peripheral $CD4^+$ T cells, but only following depletion of $CD4^+CD25^+$ T cells [19, 20]. These results suggested that NY-ESO-1–specific $CD4^+$ T-cell precursors are actually present at relatively high frequencies in healthy individuals, and that the activation/expansion of NY-ESO-1–specific naive $CD4^+$ T cells is suppressed by $CD4^+CD25^+$ Treg cells. In healthy donors and in cancer patients with NY-ESO-1–expressing tumors but without spontaneous anti-NY-ESO-1 Ab (seronegative), naturally arising NY-ESO-1–specific T-cell responses are susceptible to Treg-cell suppression and are exclusively detected from naive populations ($CD4^+CD25^-CD45RA^+$). In contrast, most NY-ESO-1–specific $CD4^+$ T cells in cancer patients with spontaneous anti-NY-ESO-1 Ab (seropositive) are derived from memory populations ($CD4^+CD25^-CD45RO^+$) and are detectable even in the presence of $CD4^+CD25^+$ Treg cells [20, 21]. After vaccination with HLA-DPB1*0401/0402-restricted NY-ESO-$1_{157-170}$ peptide in incomplete Freund's adjuvant, ovarian cancer patients develop NY-ESO-1–specific $CD4^+$ T cells with only low avidity to antigen and low sensitivity to Treg cells, even though they have an effector/memory phenotype ($CD4^+CD25^-CD45RO^+$) [21]. Still, high-avidity naive NY-ESO-1–specific T-cell precursors are present in the peripheral blood of vaccinated patients, but they are subjected to continuous $CD4^+CD25^+$ Treg-cell suppression throughout vaccination [21]. Thus, a strategy to overcome Treg-cell suppression on preexisting high-avidity naive T-cell precursors is an essential component for effective cancer vaccines.

Accumulating data shed light on recognition of pathogen-associated molecular patterns through TLRs to break the suppressive environment in tumors [22]. It has been reported that TLR stimulants, such as lipopolysaccharide or CpG, block the suppressive activity of $CD4^+CD25^+$ Treg cells partially by an IL-6–dependent mechanism [23]. TLR2 signaling was reported to stimulate the proliferation of $CD4^+CD25^+$ Treg cells and to induce temporal loss of suppressive activity of $CD4^+CD25^+$ Treg cells [24]. TLR2 signaling has also been shown to increase IL-2 secretion by effector T cells, thereby rendering them resistant to $CD4^+CD25^+$ Treg-cell–mediated suppression [25]. We and others have recently reported that vaccination of tumor antigens by TLR stimulating viral or bacterial vectors was able to not only inhibit the suppressive function of $CD4^+CD25^+$ Treg cells but also break tolerance or hyporesponsiveness of effector T cells to tumor antigens even in the presence of Treg cells [26–28].

OK-432 is a lyophilized preparation of *Streptococcus pyogenes* that binds TLR-2, TLR-4, and/or TLR-9 and activates APCs, making it attractive for potential use as an adjuvant of cancer vaccine [29–33]. OK-432–matured DCs effectively prime antigen-specific T cells in vitro [29, 34]. Importantly, OK-432 has already been used for many years as a direct anticancer agent, particularly in Japan, and has a well-established clinical safety profile. However, while it is considered that OK-432 may inhibit Treg-cell suppressive activity by stimulating several TLR signaling pathways, its influence on Treg cells has not yet been shown. In this study, we addressed whether OK-432 inhibits Treg-cell suppressive function and could be a promising adjuvant of cancer vaccines.

Results

OK-432 inhibits the suppressive activity of $CD4^+CD25^+$ Treg cells

To address whether OK-432 inhibited $CD4^+CD25^+$ Treg-cell suppression, we employed the standard in vitro suppression system. $CD4^+CD25^-$ T cells and $CD4^+CD25^{high}$ Treg cells (highest 3% of $CD4^+CD25^+$ cells) were isolated from PBMCs of healthy individuals. $CD4^+CD25^-$ T cells were cultured with irradiated autologous APCs (CD4-depleted PBMCs) and anti-CD3 Ab in the presence or absence of $CD4^+CD25^{high}$ Treg cells. $CD4^+CD25^-$ T-cell proliferation was analyzed as described in the *Materials and methods*. In accordance with previous reports [7], $CD4^+CD25^{high}$ Treg cells markedly suppressed the proliferation of $CD4^+CD25^-$ T cells (Fig. 1A and B). In sharp contrast, when OK-432 was added in the culture, suppressive activity of $CD4^+CD25^{high}$ T cells was significantly inhibited (Fig. 1A and B). In addition, OK-432 did not induce death of $CD4^+CD25^{high}$ Treg cells as the frequency of Annexin V^+ and 7-AAD^+ cells was not significantly increased in the presence of OK-432 (data not shown). Instead, $CD4^+CD25^{high}$ Treg cells exhibited marginal proliferation in the presence of

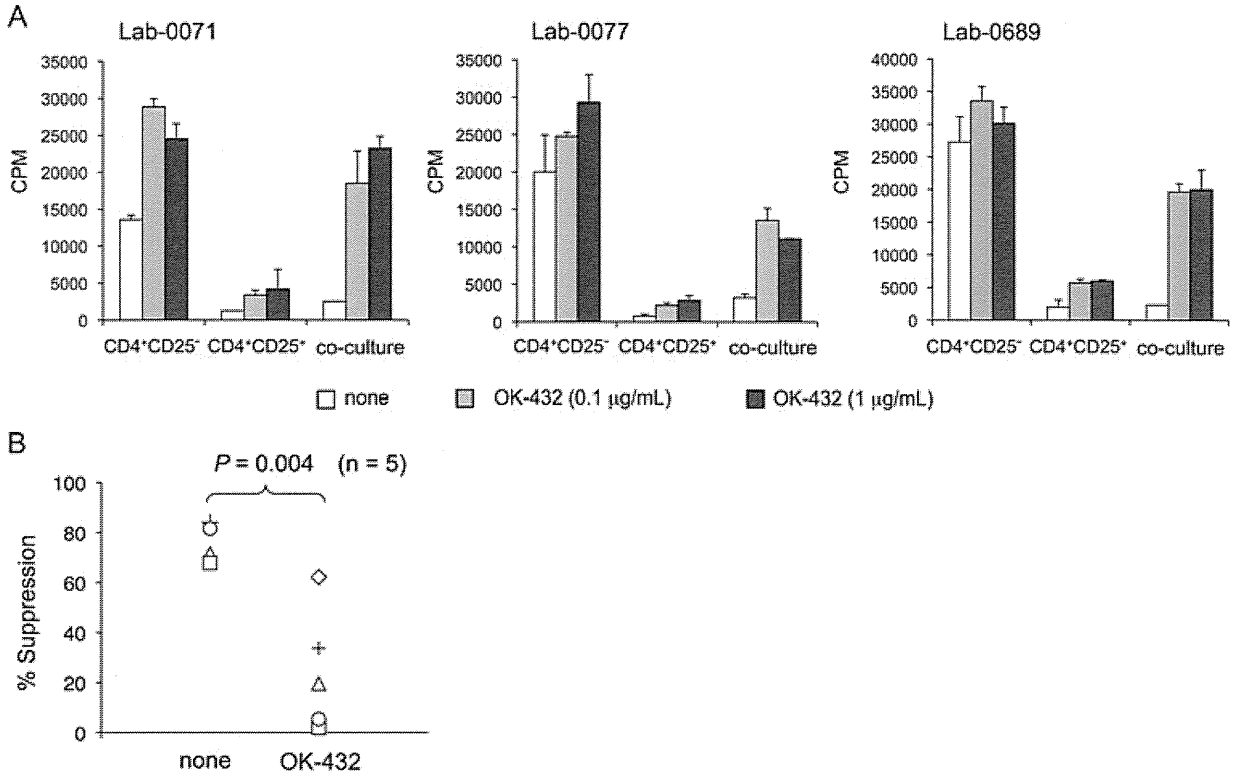

Figure 1. OK-432 overcomes Treg-cell suppression in vitro. (A, B) $CD4^+CD25^-$ T cells and $CD4^+CD25^{high}$ Treg cells were collected from PBMCs of healthy individuals as described in the *Materials and Methods*. 1×10^4 $CD4^+CD25^-$ T cells were cultured with irradiated autologous CD4-depleted PBMCs and anti-CD3 Ab in the presence or absence of 5×10^3 $CD4^+CD25^+$ Treg cells with/without OK-432. Proliferation was analyzed by ^3H-thymidine incorporation. Data of three donors are shown as mean + SD of two replicates/samples and are from one experiment representative of at least two performed. (B) Summary of percent suppression in five healthy individuals. Percent suppression was calculated as: (1 – (cpm in coculture)/(cpm in $CD4^+CD25^-$ T cells)) × 100. Data shown are from one experiment representative of at least two performed.

OK-432 (Fig. 1A). These data indicate that addition of OK-432 impairs the suppressive activity of $CD4^+CD25^{high}$ Treg cells and partially reverses anergy status of Treg cells.

Inhibition of the suppressive activity of $CD4^+CD25^+$ Treg cells by OK-432 is dependent on IL-12

Since OK-432 reportedly induces TLR-2, TLR-4, and/or TLR-9 activation and subsequent production of proinflammatory cytokines [29–33], we examined the involvement of cytokines in this inhibition of Treg-cell suppression. To this end, Abs against several candidate cytokines were added to cultures. Among cytokines tested, only blocking Ab against IL-12 significantly abrogated the inhibition of Treg-cell suppression by OK-432 (Fig. 2A).

To confirm the importance of IL-12, we next analyzed whether the addition of IL-12 could inhibit Treg-cell suppression as observed by OK-432. $CD4^+CD25^-$ T cells were cultured with $CD4^+CD25^{high}$ Treg cells, irradiated autologous APCs and anti-CD3 Ab in the presence of IL-12. Treg-cell suppressive activity was significantly inhibited by the addition of IL-12, but not IL-6 or IFN-γ (Fig. 2B). Again, IL-12 did not kill $CD4^+CD25^{high}$ Treg cells as the frequency of Annexin V^+ and 7-AAD^+ cells was not significantly increased in the presence of IL-12 (data not shown). Instead, $CD4^+CD25^{high}$ Treg cells slightly proliferated in the presence of OK-432 (Fig. 2B). These data suggest a critical role for IL-12 in the inhibition of Treg-cell suppression by OK-432.

OK-432 induces higher amounts of IL-12 but not IL-10 from APCs compared with other stimuli

To gain insight into the cellular target(s) of OK-432, we explored the origin of IL-12 after OK-432 treatment based on the essential role of IL-12 in the inhibition of Treg-cell suppression by OK-432. We then analyzed whether OK-432 stimulation indeed induced IL-12 production from APCs, such as CD3-depleted PBMCs used in the standard Treg-cell suppression assays. CD3-depleted PBMCs from healthy donors were stimulated with OK-432, LPS, or TNF-α, and cytokine production was examined. OK-432 induced significantly higher amounts of IL-12 from CD3-depleted PBMCs than LPS or TNF-α (Fig. 3A). In addition, CD3-depleted PBMCs stimulated with OK-432 induced much less IL-10 production than LPS (Fig. 3A). Similar results, i.e. IL-12 rather than IL-10 was dominantly produced by CD3-depleted PBMCs stimulated with OK-432, were obtained from four esophageal cancer patients (Fig. 3B).

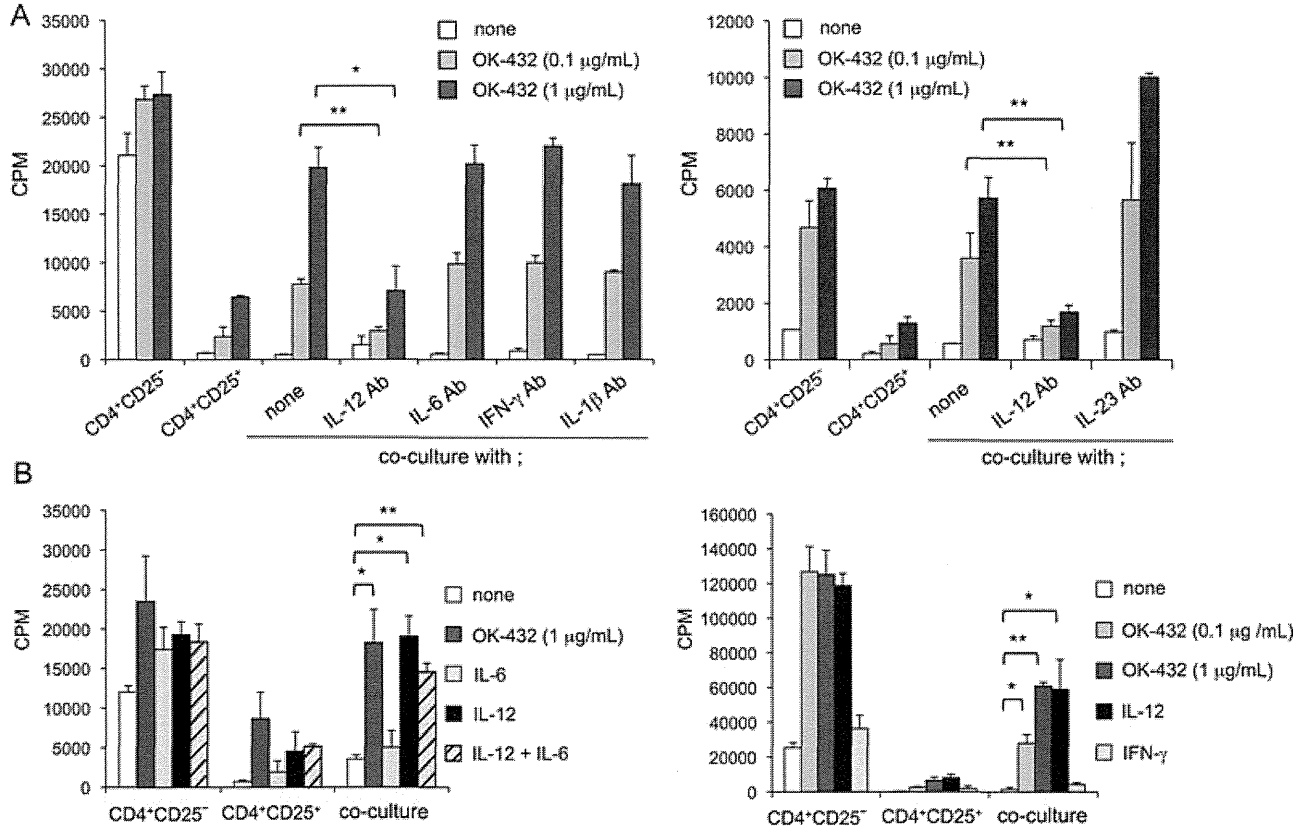

Figure 2. IL-12 is a critical cytokine to overcome Treg-cell suppression by OK-432. $CD4^+CD25^-$ T cells and $CD4^+CD25^{high}$ Treg cells were collected from PBMCs of healthy individuals. 1×10^4 $CD4^+CD25^-$ T cells were cultured with 1×10^5 irradiated autologous CD4-depleted PBMCs and anti-CD3 Ab in the presence or absence of 2.5×10^3 $CD4^+CD25^{high}$ Treg cells with/without OK-432. (A) Blocking Abs (10 μg/mL) against several cytokines as indicated were added in the culture and proliferation was measured. (B) Recombinant cytokines (IL-12; 5 ng/mL, IL-6; 5 ng/mL, IFN-γ; 100 U/mL) were added into the cultures and proliferation was analyzed. Data for one representative donor among three donors are expressed as mean + SD of two replicates/samples and are from one experiment representative of at least two performed. $^*p < 0.05$ and $^{**}p < 0.01$ as compared with control, Student's t-test.

We next examined which cell types in PBMCs produced IL-12 after OK-432 stimulation. The major sources of IL-12 in PBMCs after OK-432 stimulation were $CD11c^+$ and $CD14^+$ cells, and neither NK cells nor T cells produced IL-12 (Fig. 3C). Taken together, APCs, such as monocytes, macrophages, and DCs are considered to be the cellular targets of OK-432 to induce IL-12 which is a crucial component for the inhibition of Treg-cell suppression by OK-432.

OK-432 administration to tumor-associated exudates reduces local Treg-cell accumulation and function

As OK-432 is available as an anticancer agent in Japan and has been used for controlling tumor-associated exudate fluids by direct injection to the cavity, we next investigated its influence on Treg cells following in vivo treatment of OK-432. We analyzed the local Treg-cell accumulation and function of tumor-associated sites before and 2–3 days after local OK-432 administration. Cells were isolated from tumor-associated exudate fluids, such as pleural effusions and ascites. The frequency of Treg cells before and after treatment with OK-432 was examined by staining with Abs for CD4, CD25, and Foxp3. The $Foxp3^+$ T-cell population in $CD4^+$ T cells was markedly reduced (Fig. 4A). Furthermore, the proportion of $Foxp3^+$ T cells in $CD4^+CD25^+$ T cells was also significantly reduced after OK-432 administration (Fig. 4A and B), indicating that the balance of helper T cells to Treg cells had changed.

We next addressed the suppressive activity of $CD4^+CD25^{high}$ T cells in tumor-associated exudate fluids. $CD4^+CD25^{high}$ T cells (highest 3% gate of $CD4^+CD25^+$ cells defined with peripheral blood was applied) were isolated from tumor-associated exudate fluids and cultured with $CD4^+CD25^-$ T cells from PBMCs with irradiated autologous APCs and anti-CD3 Ab. After OK-432 administration, as the volume of tumor-associated exudate fluids decreased, sufficient amounts of $CD4^+CD25^{high}$ T cells for proliferation assays were available only from two patients. $CD4^+CD25^-$ T-cell proliferation was analyzed as described in the *Materials and Methods*. There was a trend, albeit not significant, toward a decrease in Treg-cell function after OK-432 administration (Fig. 4C). In contrast, we did not observe any differences in frequency and function of Treg cells in PBMCs

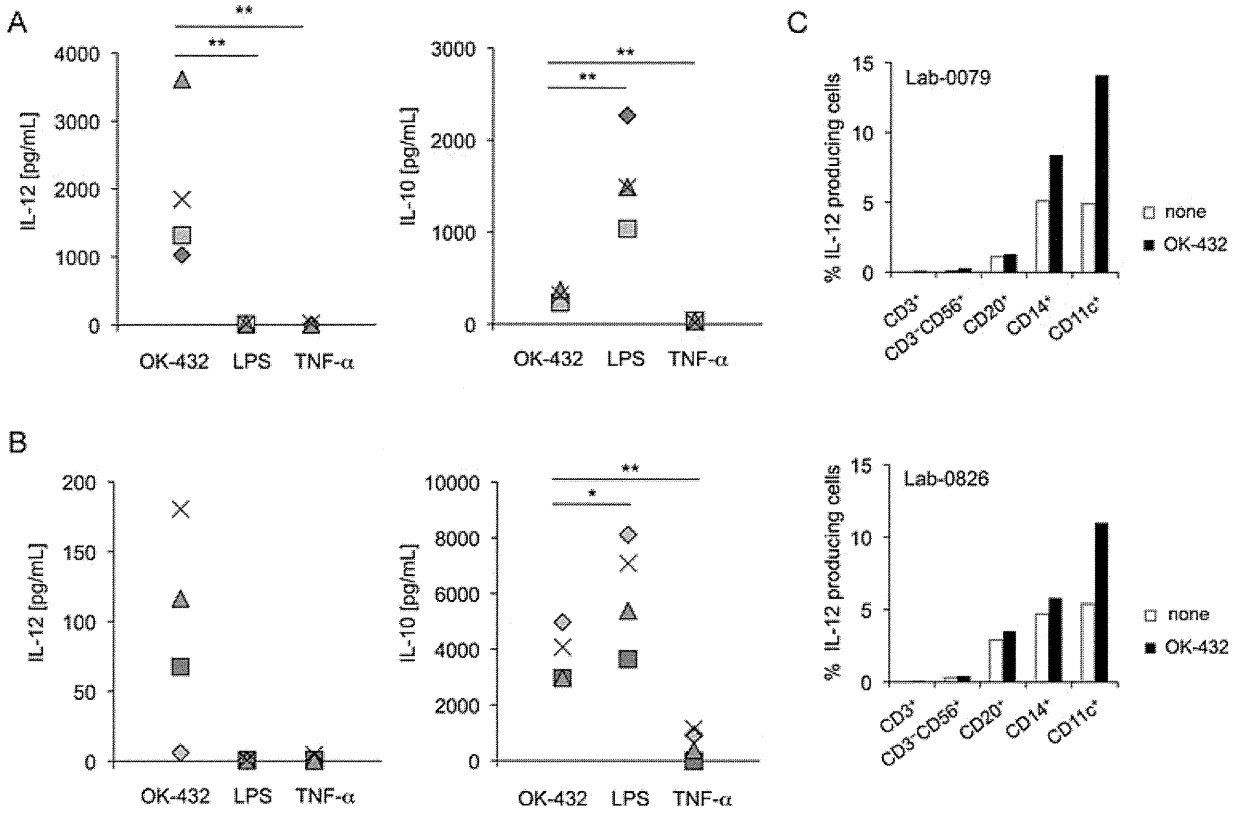

Figure 3. APCs stimulated with OK-432 produce significantly higher amounts of IL-12. (A) 1×10^5 CD3-depleted PBMCs from four healthy individuals were cultured with TNF-α (100 ng/mL), LPS (1 mg/mL), or OK-432 (1 μg/mL) and supernatant was collected 48 h later. IL-12p70 and IL-10 production was analyzed with ELISA. (B) 1×10^5 CD3-depleted PBMCs from four esophageal cancer patients were cultured as in (A) and supernatant was collected 48 h later. IL-12p70 and IL-10 production was analyzed with ELISA. (A, B) Each symbol represents an individual donor; data shown are from one experiment representative of at least two performed. (C) PBMCs from two healthy individuals were cultured with/without OK-432. Cells were subjected to staining with the indicated surface markers and then intracellular IL-12, and were analyzed by flow cytometry. Data of two donors from one experiment representative of at least two performed. $^*p < 0.05$ and $^{**}p < 0.01$ as compared with control, Student's *t*-test.

before and after OK-432 administration (data not shown). These data propose that in vivo injection of OK-432 decreases the local Treg-cell accumulation and function.

Origin of the repertoire of CD4$^+$ T-cell effectors elicited by vaccination with NY-ESO-1 and OK-432

To further explore the effect of OK-432 on the inhibition of in vivo Treg-cell activity, we also examined the potential of OK-432 as an adjuvant in a cancer vaccine. We have reported that high-avidity NY-ESO-1–specific CD4$^+$ T-cell precursors are present in naive CD45RA$^+$ populations and that their activation is rigorously suppressed by CD4$^+$CD25$^+$ Treg cells [20, 21]. We also found that synthetic peptide vaccination with incomplete Freund's adjuvant induces only peptide-specific CD4$^+$ T cells with low-avidity TCRs (recognition of >1 μM peptide but not naturally processed NY-ESO-1 protein), but not high-avidity CD4$^+$ T cells (recognition of naturally processed NY-ESO-1 protein or <0.1 μM peptide) that are susceptible to Treg-cell suppression [21]. Together, these data highlight the importance of blocking Treg-cell activity to allow activation/expansion of high-avidity NY-ESO-1–specific CD4$^+$ T-cell precursors. For this reason, we investigated whether high-avidity NY-ESO-1–specific CD4$^+$ T-cell precursors were activated by NY-ESO-1 protein vaccination with OK-432 as an adjuvant and were present in memory CD45RO$^+$ populations.

Samples from two patients who received vaccination with cholesteryl hydrophobized pullulan (CHP)-HER2 and NY-ESO-1 with OK-432 (Supporting Information Fig. 1) were available for this analysis. Whole CD4$^+$ T cells or CD4$^+$CD25$^-$CD45RO$^+$ (effector/memory) T cells before and after vaccination were presensitized with NY-ESO-1–overlapping peptides covering the entire sequence of NY-ESO-1 and specific CD4$^+$ T-cell induction was analyzed with ELISPOT assays. As the sample size was not sufficient to analyze specific CD4$^+$ T-cell induction within CD4$^+$CD25$^-$CD45RA$^+$ (naive) T cells, we analyzed whether NY-ESO-1–specific high-avidity CD4$^+$ T cells were induced from the CD4$^+$CD25$^-$CD45RO$^+$ (effector/memory) T-cell population after vaccination in Pt #1 (HLA-DR 4, 12 and HLA -DQ 4, 8) and #2 (HLA-DR 9, 15 and HLA-DQ 6, 9). Pt #1 exhibited spontaneously induced CD4$^+$ T-cell responses against NY-ESO-1_{91-110} before vaccination and the responses were maintained after extensive vaccination (Fig. 5A). These spontaneously

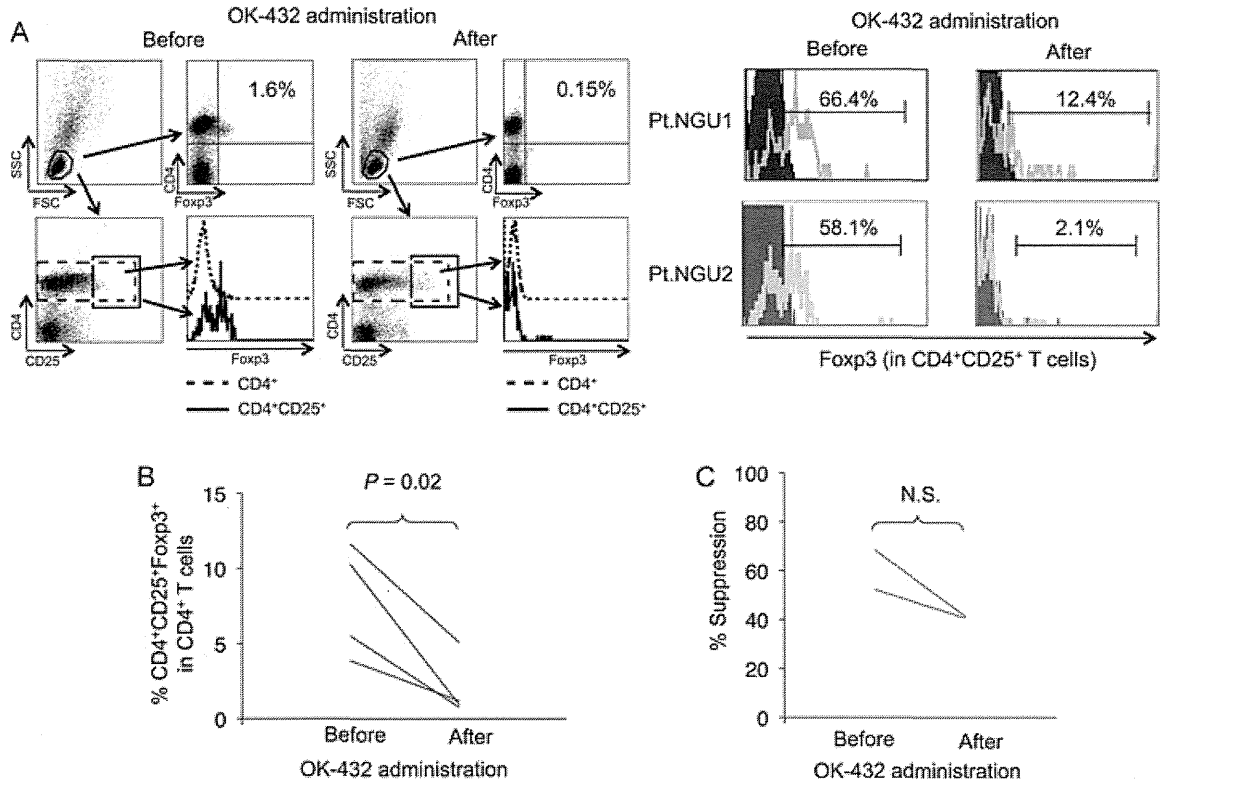

Figure 4. OK-432 administration into tumor-associated exudate fluids elicits reduction of local Treg-cell accumulation and function. Cells were isolated from tumor-associated exudate fluids (two pleural effusions or two ascites) and peripheral blood before and 2 days after OK-432 administration. (A) Cells were stained with anti-CD4, anti-CD25, and anti-Foxp3 Abs and were analyzed with flow cytometry. The staining pattern and gating method of a representative patient is shown (left); solid line histogram, Foxp3 expression in $CD4^+$ $CD25^+$ T cells; dotted line histogram, Foxp3 expression in $CD4^+$ T cells. Foxp3 expression in $CD4^+CD25^+$ T cells of two representative patients is shown (right); filled histogram, control staining; gray line histogram, Foxp3. (B) The percentage of $CD4^+CD25^+Foxp3^+$ cells in $CD4^+$ T cells is shown for $n = 4$ patients' samples. Statistical significance determined by Student's *t*-test. (C) 1×10^5 $CD4^+CD25^-$ T cells were isolated from PBMCs before OK-432 administration and cultured with irradiated autologous CD4-depleted PBMCs and anti-CD3 Ab in the presence or absence of 1×10^5 $CD4^+CD25^{high}$ T cells isolated from tumor-associated exudate fluids before and after OK-432 administration ($n = 2$). Data shown are from one experiment representative of two independent experiments.

induced NY-ESO-1_{91-110}–specific $CD4^+$ T cells were detected in the $CD4^+CD25^-CD45RO^+$ (effector/memory) T-cell population before and after vaccination. Following vaccination with NY-ESO-1 protein in the presence of OK-432, $CD4^+$ T-cell immune responses against NY-ESO-$1_{111-130}$ were newly elicited (Fig. 5A). These vaccine-induced NY-ESO-$1_{111-130}$–specific $CD4^+$ T cells were detected in the $CD4^+CD25^-CD45RO^+$ (effector/memory) T-cell population only after vaccination (Fig. 5A). In Pt #2, while specific $CD4^+$ T cells were not observed before vaccination, NY-ESO-$1_{119-141}$–specific $CD4^+$ T cells were elicited after vaccination. The vaccine-induced NY-ESO-$1_{119-141}$–specific $CD4^+$ T cells were also detected in the $CD4^+CD25^-CD45RO^+$ (effector/memory) T-cell population, as observed in Pt #1 (Fig. 5B).

NY-ESO-1 vaccination with OK-432 activates high-avidity preexisting NY-ESO-1–specific $CD4^+$ T-cells

We then asked whether vaccine-induced T cells had a high-affinity TCR that recognized naturally processed antigens [21, 28]. We established NY-ESO-1–specific $CD4^+$ T-cell clones. Four clones and a single clone that recognized different epitopes were generated from Pt #1 and Pt #2, respectively. Four minimal epitopes (NY-ESO-$1_{83-96, 94-109, 119-130, 121-134}$) were defined from $CD4^+$ T-cell clones derived from Pt #1 (Fig. 6A and data not shown). Both spontaneously induced (#2–11) and vaccine-induced (#3–1) $CD4^+$ T-cell clones recognized naturally processed NY-ESO-1 protein and as little as 0.1 nM of peptide (Fig. 6A). One minimal epitope defined from Pt #2 was NY-ESO-$1_{122-133}$ and the vaccine-induced $CD4^+$ T-cell clone (#1–1) again recognized both the naturally processed NY-ESO-1 protein and as little as 0.1 nM of peptide (Fig. 6B), indicating that these T-cell clones had high-affinity TCRs against NY-ESO-1. Together, OK-432 as an adjuvant could overcome Treg-cell suppression and activate high-affinity preexisting NY-ESO-1–specific $CD4^+$ T-cell precursors.

Discussion

While a subset of patients treated with immunotherapy has been shown to experience objective and durable clinical responses, it is becoming increasingly clear that several mechanisms

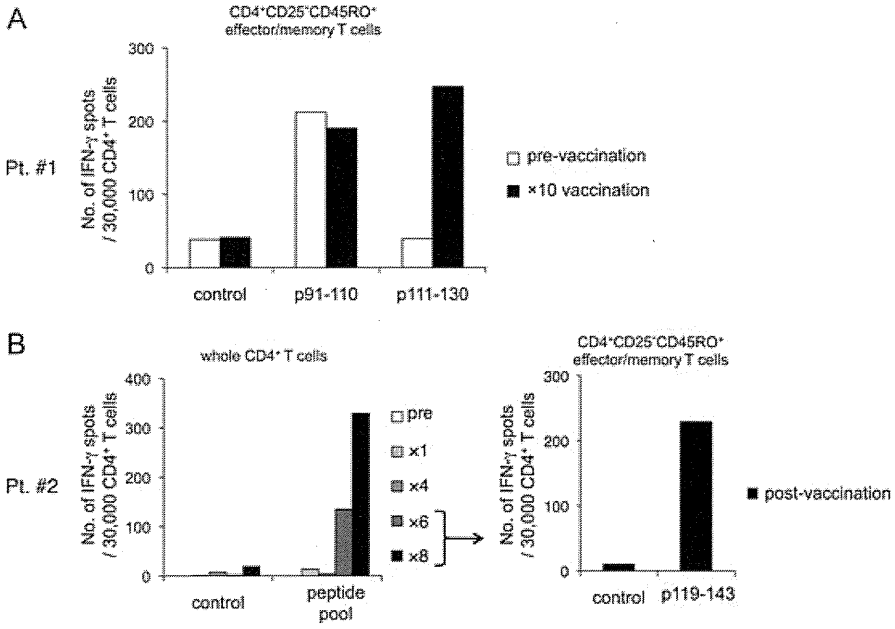

Figure 5. Vaccination with OK-432 elicits NY-ESO-1–specific effector/memory $CD4^+$ T cells $CD4^+CD25^-CD45RO^+$ T cells or unfractionated $CD4^+$ T cells were prepared from PBMCs of (A) Pt #1 (HLA-DR 4, 12 -DQ 4, 8) and (B) Pt #2 (HLA-DR 9, 15 -DQ 6, 9) before and after vaccination as described in the *Materials and Methods*; 3–5 × 10^5 $CD4^+CD25^-CD45RO^+$ T cells or unfractionated $CD4^+$ T cells were cultured with 3–5 × 10^5 CD4-depleted PBMCs pulsed with 10 μM pooled peptides covering the entire sequence of NY-ESO-1 for 3 weeks. Induction of NY-ESO-1–specific IFN-γ production was analyzed by ELISPOT. Data shown are from one experiment representative of at least two experiments performed.

downregulate antitumor immunity during the course of the immune response and play a major role in limiting the effectiveness of cancer immunity [6, 35, 36]. A plethora of cell types, cell surface molecules, and soluble factors mediate this suppressive activity [3, 6, 35, 36]. Among them, $CD4^+CD25^+Foxp3^+$ Treg cells play a crucial role by suppressing a wide variety of immune responses, and finding ways to control Treg-cell suppression is a major priority in this field [6, 7]. In this study, we showed the potential of OK-432 (a penicillin-inactivated and lyophilized preparation of *Streptococcus pyrogenes*) which stimulates TLR signals [30, 33, 34] to control Treg-cell suppression, supporting the idea that OK-432 may be a promising adjuvant for cancer vaccines by inhibiting Treg-cell suppression and by augmenting induction of tumor-specific T cells against coadministered protein antigens.

Appropriate adjuvant combinations, such as those that are MyD88-dependent or MyD88-independent, or those that are TRIF-coupled and include endosomal signals, are known to synergistically activate DCs with regard to the production of inflammatory cytokines [37, 38]. As OK-432 is derived from bacterial components, its capacity to bind a combination of various TLRs makes it attractive. It has been shown that OK-432 exhibits antitumor effects through TLR-2, TLR-4, and TLR-9 using knockout mice for each TLR [30, 33, 34]. Alternatively, OK-432 reportedly stimulates DCs through the β_2-integrin system rather than via TLR signals [29]. In the presence of OK-432, Treg cells slightly proliferated with TCR stimulation. TLR2 triggering results in a temporary loss of the anergic status of Treg cells and is associated with loss of Treg-cell suppressive function [24, 25]. The perturbation of Treg-cell anergy by OK-432 through TLR2 stimulation may play a role, at least in part, in the inhibition of Treg-cell suppressive function.

In accordance with previous reports [29, 34], we showed that APCs, including $CD11c^+$ and $CD14^+$ cells (monocytes, macrophage, and DCs), stimulated with OK-432 exhibited significantly higher production of IL-12 as compared with that of LPS- or TNF-α–matured APCs, and that OK-432–induced IL-12 from these APCs was a critical component for abrogating Treg-cell activity. Additionally, we found that monocyte-derived DCs stimulated with OK-432 produced significantly higher amounts of IL-12 compared with DCs stimulated with LPS or TNF-α (Supporting Information Fig. 2). It has been reported that IL-12 receptor expressed on effector T cells, but not on Treg cells has a critical role for abrogating Treg-cell suppression by IL-12 in mice [39, 40]. In accordance with this, downregulation of IL-12 receptors by siRNA on effector cells partially abrogated the OK-432–induced inhibition of Treg-cell suppressive activity (Supporting Information Fig. 3). IL-12 receptor was induced in both effector T cells and Treg cells after activation (Supporting Information Fig. 3). We attempted to downregulate the IL-12 receptor on Treg cells with siRNA to explore the exact target(s) of IL-12, however, the limitation in the availability of human materials hampered these analyses. Thus, IL-12 produced by APCs on the OK-432 stimulation could have two (or more) mutually compatible activities, (i) rendering effector cells resistant to Treg-cell suppression and (ii) inhibiting Treg-cell suppressive function directly, though the in vivo data argue against direct inhibition of Treg-cell suppression [39, 40].

Local administration of OK-432 reduced the number of $CD4^+CD25^+Foxp3^+$ Treg cells in tumor-associated exudate fluids. After administration of OK-432, local chemokine gradient may be changed and infiltration of Treg cells may be blocked [6, 13]. Alternatively, the inflammatory environment after OK-432 administration may be suitable for effector T-cell activation and IL-2, that is critical for Treg-cell survival and function [41], may not be adequately provided, as observed during severe *Toxoplasma gondii* infection [42]. In addition, suppressive function of $CD4^+CD25^{high}$ T cells in tumor-associated

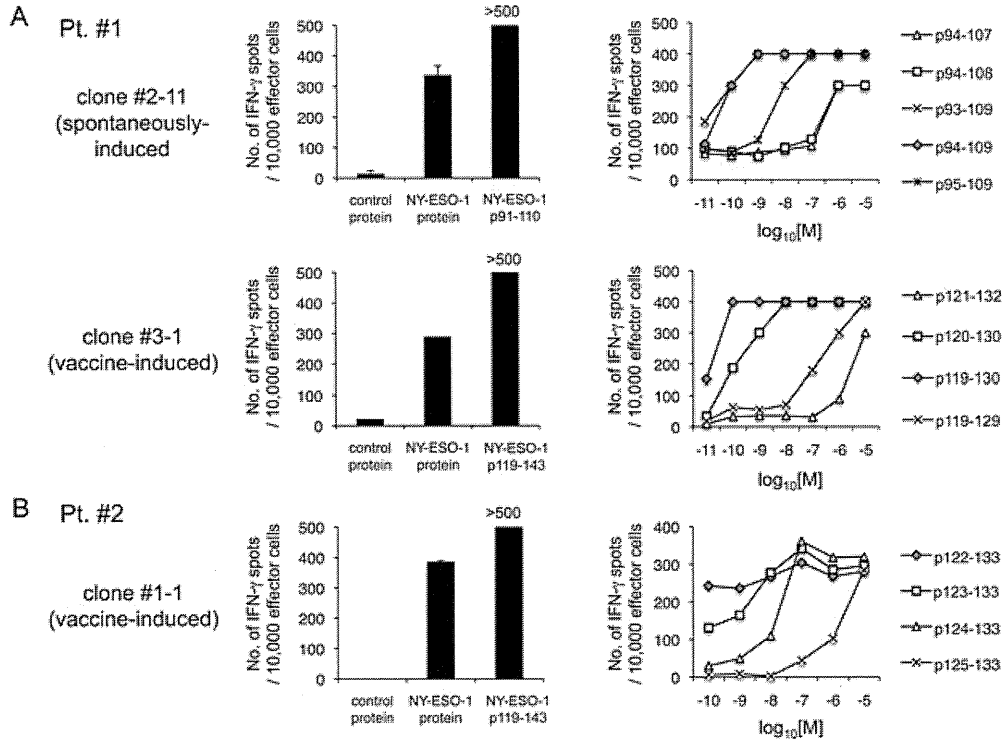

Figure 6. Vaccination with OK-432 activates high-avidity NY-ESO-1–specific CD4$^+$ T cells. NY-ESO-1–specific CD4$^+$ T-cell clones were generated from PBMCs of patients harboring NY-ESO-1–specific CD4$^+$ T cells. CD4$^+$ T-cell clones from (A) Pt. #1 and (B) Pt. #2 were stimulated and expanded by 30 ng/mL anti-CD3 Ab in the presence of 20 U/mL IL-2 and irradiated PBMCs and EBV-transformed human B lymphocytes as feeder cells. A couple of weeks later, these NY-ESO-1–specific CD4$^+$ T cell clones (1×10^4/well) were cultured with 1×10^4 NY-ESO-1 protein-pulsed DCs or 2×10^4 EBV-B cells pulsed with graded amounts of peptides and NY-ESO-1–specific IFN-γ production was analyzed by ELISPOT. Data are shown as mean ± SD of two replicates and are from one experiments representative of at least two experiments performed.

exudate fluids was reduced after OK-432 treatment in accordance with decreased expression of Foxp3 [43]. Considering the fact that IL-12, a main effector molecule induced by OK-432, renders effector cells resistant to Treg-cell suppression, direct administration of OK-432 may change the immunological balance in the local microenvironment from suppression by Treg cells to activation of helper T cells by augmenting helper T-cell activity. However, the sample size of patients analyzed in this study was relatively small and warrants cautious interpretation.

We have previously shown that while naive NY-ESO-1–specific CD4$^+$ T-cell precursors are present in wide range of healthy individuals and cancer patients, their activation is kept under stringent CD4$^+$CD25$^+$ Treg-cell control [20, 21, 28]. Using OK-432 as an adjuvant, we detected high-affinity NY-ESO-1–specific CD4$^+$ T cells in effector/memory population after vaccination in two esophageal cancer patients. In Pt #1, we found two responses; spontaneous and vaccine-induced NY-ESO-1–specific CD4$^+$ T cells. Both of them exhibited a similar efficiency to recognize titrated peptide, indicating that these NY-ESO-1–specific CD4$^+$ T cells had TCRs with similar affinity and were likely activated from naive high-affinity NY-ESO-1–specific CD4$^+$ T-cell precursors. Vaccination with minimal peptide in incomplete Freund's adjuvant fails to activate high-affinity NY-ESO-1–specific CD4$^+$ T-cell precursors, rather it dominantly expands low-avidity effector/memory CD4$^+$ T cells that cannot recognize naturally processed antigens [21]. In addition, following DNA vaccination covering the entire sequence of NY-ESO-1, high-avidity NY-ESO-1–specific CD4$^+$ T cells were not detected persistently because of rapid suppression by Treg cells [44]. While these data suggest a critical role for the inhibition of Treg-cell suppression by OK-432 in the activation of high-affinity NY-ESO-1–specific CD4$^+$ T-cell precursors, it is still difficult to obtain conclusive evidence without direct in vivo Treg-cell inhibition/depletion. To formally address this issue, clinical trials using Treg-cell depletion reagents and another clinical trial having two arms of patients receiving NY-ESO-1 with/without OK-432 would be required.

Certain types of immunization methods or DC stimulations elicit/augment CD4$^+$CD25$^+$ Treg cells in vivo [10–12, 45]. As many tumor-associated antigens recognized by autologous tumor-reactive lymphocytes are antigenically normal self-constituents [1–3], they also could be recognized with CD4$^+$CD25$^+$ Treg cells. Given that a proportion of cancer/testis antigens are targets of Treg cells [46], it is necessary to avoid unwanted activation of cancer/testis antigen-specific CD4$^+$CD25$^+$ Treg cells. Though the sample size of patients analyzed in this study was small and warrants cautious interpretation, including OK-432 in vaccine components as an adjuvant would be a promising strategy to establish favorable circumstances for stimulating effector T cells by inhibiting Treg-cell activation. Furthermore, since this agent has a long history and is widely applied as an anticancer drug, particularly

in Japan, its clinical safety profile has been already established. Our data provide a critical cue for effective cancer vaccines and immunotherapy during antigen priming through modulation of $CD4^+CD25^+$ Treg-cell function.

Materials and methods

Blood samples

All healthy donors were subjects with no history of autoimmune disease. PBMCs, pleural effusions, or ascites from cancer patients were collected before and after local administration of OK-432 based on the protocol approved by the Human Ethics Committees of Mie University Graduate School of Medicine and Nagasaki University Graduate School of Medicine. PBMCs from esophageal cancer patients enrolled in a clinical trial of CHP-NY-ESO-1 and CHP-HER2 vaccination with OK-432 [47] (Supporting Information Fig. 1) were collected based on the protocol approved by the Human Ethics Committees of Mie University Graduate School of Medicine and Kitano Hospital. The clinical trial was conducted in full conformity with the current version of the Declaration of Helsinki and was registered as NCT00291473 of Clinical Trial.gov, and 000001081 of UMIN Clinical Trial Registry. All samples were collected after written informed consent.

Abs and reagents

Synthetic peptides of NY-ESO-1_{1-20} (MQAEGRGTGGSTGDADGPGG), NY-ESO-1_{11-30} (STGDADGPGGPGIPDGPGGN), NY-ESO-1_{21-40} (PGIPDGPGGNAGGPGEAGAT), NY-ESO-1_{31-50} (AGGPGEAGATGGRGPRGAGA), NY-ESO-1_{41-60} (GGRGPRGAGAARASGPGGGA), NY-ESO-1_{51-70} (ARASGPGGGAPRGPHGGAAS), NY-ESO-1_{61-80} (PRGPHGGAASGLNGCCRCGA), NY-ESO-1_{71-90} (GLNGCCRCGARGPESRLLEF), NY-ESO-1_{81-100} (RGPESRLLEFYLAMPFATPM), NY-ESO-1_{91-110} (YLAMPFATPMEAELARRSLA), NY-ESO-$1_{101-120}$ (EAELARRSLAQDAPPLPVPG), NY-ESO-$1_{111-130}$ (QDAPPLPVPGVLLKEFTVSG), NY-ESO-$1_{119-143}$ (PGVLLKEFTVSGNILTIRLTAADHR), NY-ESO-$1_{131-150}$ (NILTIRLTAADHRQLQLSIS), NY-ESO-$1_{139-160}$ (AADHRQLQLSISSCLQQLSLLM), NY-ESO-$1_{151-170}$ (SCLQQLSLLMWITQCFLPVF), NY-ESO-$1_{161-180}$ (WITQCFLPVFLAQPPSGQRR), and HIV $P17_{37-51}$ (ASRELERFAVNPGLL) [48] were obtained from Invitrogen (Carlsbad, CA, USA). Recombinant NY-ESO-1 protein was prepared using similar procedures as described previously [49]. OK-432 was purchased from Chugai Pharmaceutical (Tokyo, Japan). LPS (*Escherichia coli* 055:B5) was obtained from Sigma (St. Louis, MO, USA). Purified and FITC-conjugated anti-IL-12 (C8.6; mouse IgG1), purified anti-IL-6 (MQ2–13A5; rat IgG1), purified anti-IFN-γ (NIB42; mouse IgG1), purified anti-IL-23 (HNU2319; mouse IgG1), PE-conjugated anti-CD20 (2H7; mouse IgG2b) and PE-conjugated anti-CD56 (MEM188; mouse IgG2a) Abs were purchased from eBioscience (San Diego, CA, USA). Purified anti-IL-1β Ab (8516; mouse IgG1) was purchased from R&D Systems (Minneapolis, MN, USA). PE-conjugated anti-CD14 (MφP9; mouse IgG2b), PE-conjugated anti-CD45RA (HI100; mouse IgG2b), PerCP-conjugated anti-CD4 (RPA-T4; mouse IgG1), and FITC-conjugated anti-CD4 (RPA-T4; mouse IgG1), Foxp3 (259D; mouse IgG1), and CD45RO (UCHL1; mouse IgG2a) Abs were purchased from BD Biosciences (Franklin Lakes, NJ, USA). PerCP-Cy5.5-conjugated anti-CD11c Ab (3.9; mouse IgG1) was obtained from Biolegend (San Diego CA, USA). PE-conjugated anti-CD25 Ab (4E3; mouse IgG2b) was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Recombinant IL-6, IL-12, and TNF-α were purchased from PeproTech (Rocky Hill, NJ, USA).

Intracellular cytokine staining

PBMCs were cultured with/without OK-432 and GolgiStop reagent (BD Biosciences) for 20 h. Cells were stained for cell surface markers and then for intracellular cytokine (IL-12) after permeabilization. Results were analyzed by flow cytometry (FACSCanto; BD Biosciences).

Generation of NY-ESO-1–specific $CD4^+$ T cells

NY-ESO-1–specific $CD4^+$ T cells were elicited as described previously [20]. Briefly, $CD4^+$ T cells and $CD4^+CD25^-$ T cells were isolated from PBMCs using a $CD4^+CD25^+$ Treg Isolation Kit (Miltenyi Biotec). $CD4^+CD25^-$ T cells were further separated into $CD45RO^+$ T cells or $CD45RA^+$ T cells by FACSAria (BD Bioscience) after staining with anti-CD45RO and CD45RA Abs. $CD4^-$ PBMCs pulsed with 10 μM of peptide overnight were used as APCs. After irradiation, 5×10^5 APCs were added to round-bottom 96-well plates (Nunc, Roskilde, Denmark) containing $1–5 \times 10^5$ unfractionated $CD4^+$ or $CD4^+CD25^-CD45RO^+$ T cells and were fed with 10 U/mL IL-2 (Kindly provided by Takeda Pharmaceutical, Osaka, Japan) and 20 ng/mL IL-7 (R&D Systems). Subsequently, one-half of medium was replaced by fresh medium containing IL-2 (20 U/ml) and IL-7 (40 ng/mL) twice per week.

Generation of NY-ESO-1–specific $CD4^+$ T-cell clones

Cloning was performed by limited dilution as described previously [50]. Briefly, NY-ESO-1–specific $CD4^+$ T cell lines (0.3 cells/well) were stimulated and expanded in the presence of irradiated 5×10^4 cells/well PBMCs and 1×10^4 cells/well irradiated EBV-transformed human B lymphocytes with 10% AB serum, 20 U/ml IL-2, and 30 ng/mL anti-CD3 Ab (OKT3; eBioscience) in 96-well round-bottom plates.

Proliferation assay

$CD4^+CD25^-$ T cells were cultured with 1×10^5 irradiated CD4-depleted PBMCs and stimulated with 0.5 μg/mL anti-CD3 Ab

(OKT3, eBioscience) in round-bottom 96-well plates. $CD4^+CD25^{high}$ Treg cells (highest 3% of $CD4^+CD25^+$ cells) were purified with FACSAria (BD Biosciences), and graded numbers of them added in the culture as indicated in figure legends. Proliferation was evaluated by ^{3}H-thymidine with 1 μCi/well for the last 18 h of 6-day culture. ^{3}H-thymidine incorporation was measured by a scintillation counter.

ELISPOT (enzyme-linked immunospot) assay

The number of IFN-γ secreting antigen-specific $CD4^+$ T cells was assessed by ELISPOT assays as described [20, 21]. Briefly, flat-bottomed, 96-well nitrocellulose-coated microtiter plates (Millipore, Bedford, MA, USA) were coated with anti-IFN-γ Ab (1-D1K; MABTECH, Stockholm, Sweden). The presensitized T cells and phytohaemagglutinin (PHA HA15; Murex Diagnostics, Dartford, UK) activated $CD4^+$ T cells, EBV-transformed human B lymphocytes or DCs pulsed with 10 μM of peptides or 25 μg/mL protein overnight were added to each well and incubated for 24 h. Spots were developed using biotinylated anti-IFN-γ Ab (7-B6–1-biotin; MABTECH), alkaline phosphatase conjugated streptavidin (Roche, Mannheim, Germany) and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma) and counted with C.T.L. Immunospot analyzer and software (Cellular Technologies, Cleveland, OH, USA).

Preparation of monocyte-derived DCs

Monocyte-derived DCs were generated from PBMCs as previously described with some modifications [51]. Briefly, $CD14^+$ monocytes were enriched by positive selection using CD14 Microbeads (Miltenyi Biotec). Monocytes were cultured in the presence of 20 ng/mL GM-CSF (Immunex, Seattle, WA, USA) and 20 ng/mL IL-4 (R&D systems) in RPMI1640 supplemented with 2.5% fetal calf serum. Medium was replaced by fresh medium containing cytokines 3 days later. On day 6, cells were harvested and used for subsequent experiments.

ELISA

The concentration of IL-12p70 and IL-10 was measured by ELISA Kit (eBioscicence) according to the instruction provided by the manufacturer.

Statistical analysis

Statistical significance was evaluated by Student's *t*-test; *p* values less than 0.05 are considered significant.

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Abbreviation: **CHP**: cholesteryl hydrophobized pullulan

Full correspondence: Dr. Hiroyoshi Nishikawa, Experimental Immunology, Immunology Frontier Research Center, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan
Fax: +81-6-6879-4464
e-mail: nisihiro@ifrec.osaka-u.ac.jp

Additional correspondence: Sacha Gnjatic, Ludwig Institute for Cancer Research, New York Branch, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10065, USA.
e-mail: gnjatics@mskcc.org

Additional correspondence: Hiroshi Shiku, Departments of Cancer Vaccine and Immuno-Gene Therapy, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan.
e-mail: shiku@clin.medic.mie-u.ac.jp

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Article

A novel human-derived antibody against NY-ESO-1 improves the efficacy of chemotherapy

Anurag Gupta[1], Natko Nuber[1,6], Christoph Esslinger[2], Mareike Wittenbrink[2], Martin Treder[2], Alexandro Landshammer[1], Takuro Noguchi[3], Marcus Kelly[3], Sacha Gnjatic[3], Erika Ritter[3], Lotta von Boehmer[1], Hiroyoshi Nishikawa[4], Hiroshi Shiku[5], Lloyd Old[3], Gerd Ritter[3], Alexander Knuth[1] and Maries van den Broek[1]

[1]*Department of Oncology, University Hospital Zürich, Zürich, Switzerland*
[2]*CT-Atlantic, Schlieren, Switzerland*
[3]*Ludwig Institute for Cancer Research, New York Branch, Memorial Sloan-Kettering Cancer Center, New York, NY, USA*
[4]*Experimental Immunology, Immunology Frontier Research Center, Osaka University, Osaka, Japan*
[5]*Departments of Cancer Vaccine and Immuno-Gene Therapy, Mie University Graduate School of Medicine, Tsu, Mie, Japan*
[6]*Department of Biomedicine, University of Basel, Basel, Switzerland*

We investigated whether antibodies against intracellular tumor-associated antigens support tumor-specific immunity when administered together with a treatment that destroys the tumor. We propose that released antigens form immune complexes with the antibodies, which are then efficiently taken up by dendritic cells. We cloned the first human monoclonal antibodies against the Cancer/Testis (CT) antigen, NY-ESO-1. We tested whether the monoclonal anti-NY-ESO-1 antibody (12D7) facilitates cross-presentation of a NY-ESO-1-derived epitope by dendritic cells to human CD8+ T cells, and whether this results in the maturation of dendritic cells *in vitro*. We investigated the efficacy of 12D7 in combination with chemotherapy using BALB/c mice bearing syngeneic CT26 tumors that express intracellular NY-ESO-1. Human dendritic cells that were incubated with NY-ESO-1:12D7 immune complexes efficiently stimulated NY-ESO-$1_{157-165}$/HLA-A2-specific human CD8+ T cells to produce interferon-γ, whereas NY-ESO-1 alone did not. Furthermore, the incubation of dendritic cells with NY-ESO-1:12D7 immune complexes resulted in the maturation of dendritic cells. Treatment of BALB/c mice that bear CT26/NY-ESO-1 tumors with 5-fluorouracil (5-FU) plus 12D7 was significantly more effective than chemotherapy alone. We propose systemic injection of monoclonal antibodies (mAbs) against tumor-associated antigens plus a treatment that promotes the local release of those antigens resulting in immune complex formation as a novel therapeutic modality for cancer.

Keywords: NY-ESO-1, antibody, chemotherapy

Introduction

Cancer/Testis (CT) antigens form an extended family of proteins that are frequently expressed in a large variety of malignancies but are absent from healthy tissue, except for the testis and placenta. Cancer patients often develop spontaneous immune responses toward CT antigens, which illustrate their immunogenicity (1-3). Their apparent immunogenicity and unique expression pattern make CT antigens attractive targets for immunotherapy, and a number of clinical trials in which cancer patients were immunized with CT antigens in different forms have been completed, some of which show objective clinical responses (4-12).

Dendritic cell (DC) maturation is a key prerequisite for the activation of T cells, and moreover, antigen presentation by steady-state DCs results in peripheral tolerance induction, a process that is considered crucial for the protection against autoimmunity (13, 14). DC maturation usually is induced by infection or inflammation—or by adjuvants for that matter—and can be a local event. Insufficient maturation of tumor-associated DCs may be one of multiple reasons for the compromised response of tumor-infiltrating T cells compared to peripheral T cells (15, 16). Cross-presentation of sufficient amounts of tumor-derived antigens may be another limiting factor, especially because the number of tumor-associated DCs often is low and cross-presentation is inefficient (17, 18). Therefore, we developed a novel immunotherapeutic approach that combines enhanced cross-presentation of epitopes derived from intracellular proteins with concomitant DC maturation. We hypothesized that administration of monoclonal antibodies (mAbs) against CT antigens together with a therapy that releases these usually intracellular antigens may support the local formation of immune complexes, which are efficiently taken up by DCs (19, 20) resulting in increased presentation of CT antigen-derived epitopes to CD8+ T cells. Because there is evidence that the uptake of immune complexes by DCs through the activating receptor for IgG (FcγRIIA) results in DC maturation (21), the use of mAbs against CT antigens may serve both purposes: DC activation and enhanced cross-presentation.

The fact that NY-ESO-1 is one of the best-characterized and most immunogenic CT antigens known to date (22, 23) and is frequently expressed by tumors of different origin (6, 24) prompted us to clone human-derived mAbs against NY-ESO-1 from patients who had high serum levels of NY-ESO-1-specific IgG and, thus, presumably a high frequency of NY-ESO-1-specific B cells. The obvious advantage of cloning a therapeutic antibody from humans is that adverse side effects of such an antibody are very unlikely and that it therefore can relatively be quickly tested in clinical trials. We report here the generation of the first human-derived IgG1 mAbs against NY-ESO-1 and the selection of a lead development candidate (12D7). We show that 12D7 facilitates cross-presentation of a NY-ESO-1-derived epitope to CD8+ T cells, that 12D7:NY-ESO-1 immune

complexes induce the maturation of human monocyte-derived DCs *in vitro*, and that 12D7 significantly enhances the therapeutic efficacy of chemotherapy using a preclinical syngeneic mouse model.

Table 1

Binding of human monoclonal anti-NY-ESO-1 antibodies to NY-ESO-1. Comparison of EC_{50} and equilibrium affinity constants for the binding between NY-ESO-1 and different anti-NY-ESO-1 antibodies.

Antibody	EC_{50} [pM] (prok. NY-ESO-1)	K_D [M] (prok. NY-ESO-1)	K_D [M] (euk. NY-ESO-1)
12D7	1.14	$2.08x10^{-10}$	$1.56x10^{-10}$
1D4	2.23	$1.62x10^{-9}$	$2.24x10^{-10}$
30D6	1.09	$4.35x10^{-9}$	$2.65x10^{-9}$
31E4	9.52	$1.9x10^{-8}$	$2.23x10^{-8}$
15B12	72.6	---	---
E978 control	6.66	$2.56x10^{-8}$	$1.56x10^{-11}$

Results

Cloning of human-derived monoclonal antibodies from cancer patients

We cloned eight different NY-ESO-1-specific human-derived monoclonal antibodies (HD mAbs) from a melanoma patient, of which the following five were selected for further analysis based on their affinity to the target: 1D4, 12D7, 15B12, 30D6, and 31E4. All HD mAbs were of IgG1 isotype.

In vitro characterization of HD mAbs

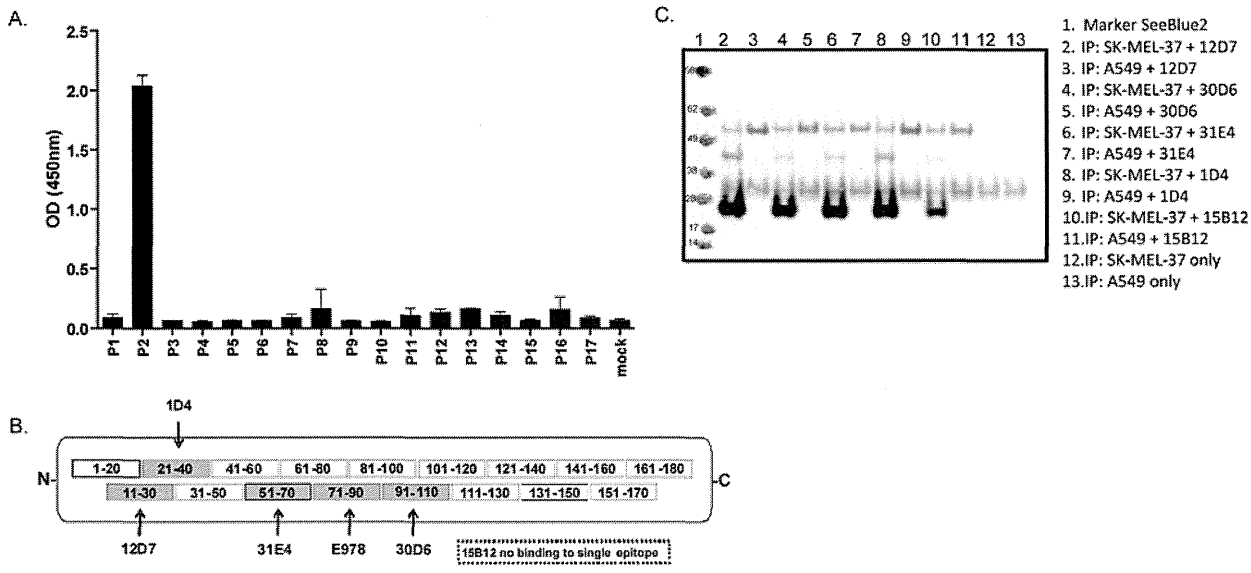
To compare the binding properties of five different anti-NY-ESO-1 HD mAbs to recombinant NY-ESO-1 protein, we determined the half-maximal effective concentration (EC_{50}) using a protein ELISA. All antibodies bound recombinant NY-ESO-1 produced in bacteria in the low pM range. Actual binding constants to recombinant NY-ESO-1 produced in bacteria and in eukaryotic cells were determined by surface plasmon resonance (Biacore Systems) (Table 1).

To determine the epitopes recognized by the different mAbs, we used a set of overlapping peptides spanning the complete NY-ESO-1 protein as coating antigen in ELISA. As shown in Figure 1A, 12D7 binds to a peptide representing the amino acids 11 to 30 from the NY-ESO-1 protein, but not to the two adjacent peptides that span amino acids 1-20 or 21-40. This suggests that the epitope recognized by 12D7 lies at the junction of these two peptides around amino acid 20 of NY-ESO-1. Figure 1B summarizes the epitope-specificity of all five anti-NY-ESO-1 antibodies. In addition, all antibodies were tested for binding to endogenous NY-ESO-1 from the human melanoma cell line SK-MEL-37 by immunoprecipitation. All antibodies precipitate NY-ESO-1 from a cell lysate of an NY-ESO-1+ cell line (SK-MEL-37) (Figure 1C). Because 12D7 had the highest affinity for eukaryotic NY-ESO-1, we performed further experiments with this mAb.

12D7 facilitates cross-presentation of NY-ESO-1 by DCs and induces concomitant DC maturation

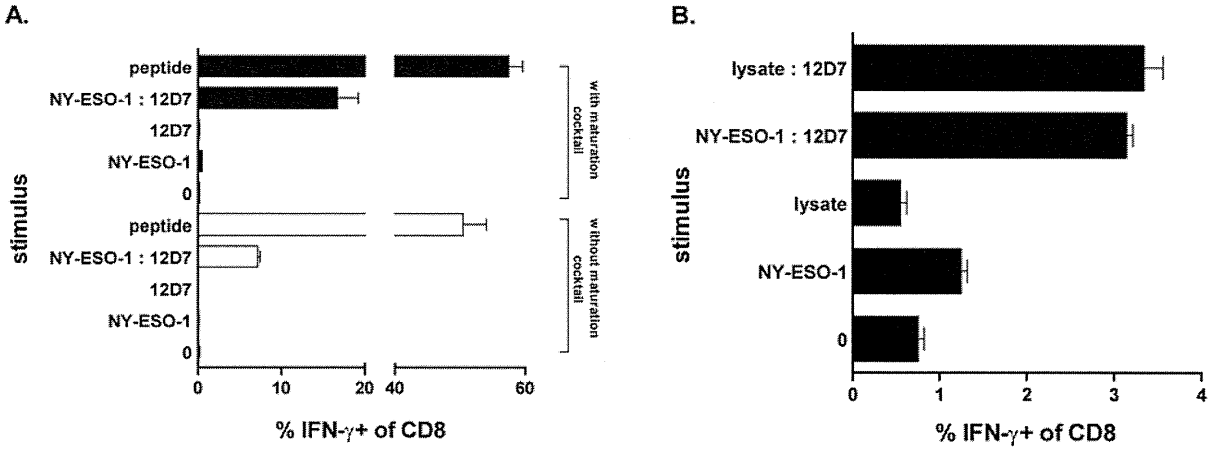
To test whether 12D7 facilitates the cross-presentation of NY-ESO-1-derived epitopes *in vitro*, we generated monocyte-derived, HLA-A*0201+ DCs and fed them with 12D7:NY-ESO-1 immune complexes, NY-ESO-1, or 12D7. DCs were subsequently incubated with cloned NY-ESO-$1_{157-165}$/HLA-A*0201-specific CD8+ T cells, and the percentage of T cells that produced IFN-γ was used as readout for antigen recognition. Mature DCs fed with NY-ESO-1 protein induced IFN-γ production in a low but discernible percentage of T cells (Figure 2A, black bars), which did not occur when DCs were not matured (Figure 2A, white bars). DCs fed with 12D7:NY-ESO-1 immune complexes induced the production of IFN-γ in a much

Figure 1



Epitope mapping of anti-NY-ESO-1 human monoclonal antibodies. (A) Representative peptide ELISA for antibody 12D7, where P1-P17 represent overlapping NY-ESO-1 peptides. (B) Overview of the specificities of different NY-ESO-1 specific human-derived mAbs. (C) Immunoprecipitation of NY-ESO-1 from a cell lysate of a NY-ESO-1+ cell line SK-MEL-37 or a NY-ESO-1- cell line A549 by human anti-NY-ESO-1 mAbs.

Figure 2



Human monoclonal anti-NY-ESO-1 antibody (12D7) facilitates cross-presentation of a NY-ESO-1-derived, HLA-A2-restriced epitope (NY-ESO-$1_{157-165}$). (A) HLA-A2+, monocyte-derived DCs were incubated with 20 μg NY-ESO-1 protein, 200 μg human monoclonal anti-NY-ESO-1 antibody (12D7), with immune complexes (12D7:NY-ESO-1) or with media for 3 h, were washed and cultured for 36 h with (black bars) or without (white bars) 25 ng/mL TNF-α + 1 μg/mL sCD40L (maturation cocktail). 6 x 10^4 cloned, NY-ESO-$1_{157-165}$ /HLA-A2-specific CD8+ T cells were added to 10^5 DCs in the presence of 10 μg/mL Brefeldin A, followed by a 5 h incubation and subsequent surface staining for CD8 and intracellular staining for IFN-γ. 10^{-6} M peptide was added to DCs as positive control. All cultures were performed in triplicate. (B) HLA-A2+, monocyte-derived DCs were incubated with 20 μg NY-ESO-1 protein, 200 μg human monoclonal anti-NY-ESO-1 antibody (12D7), with lysate of 10^7 NY-ESO-1+ SK-MEL-37 cells (lysate), with immune complexes (NY-ESO-1:12D7 or lysate:12D7), or with media (0) for 3 h, were washed and cultured for 36 h with 25 ng/mL TNF-α + 1 μg/mL sCD40L (maturation cocktail). 6 x 10^4 cloned, NY-ESO-$1_{157-165}$ /HLA-A2-specific CD8+ T cells were added to 10^5 DCs in the presence of 10 μg/mL Brefeldin A, followed by a 5 h incubation and subsequent surface staining for CD8 and intracellular staining for IFN-γ. All cultures were performed at least in duplicate.

higher percentage of T cells and, importantly, also did so when DCs that were not deliberately matured were used (Figure 2A, compare black and white bars). None of the negative controls—DCs fed with 12D7, mock immune complexes, or medium—induced IFN-γ production (Figure 2A and data not shown). To exclude that our observations are a peculiarity of recombinant NY-ESO-1, we incubated 12D7 with a cell lysate of SK-MEL-37 cells, which naturally express NY-ESO-1, and subsequently fed this mixture to DCs. DCs fed with the 12D7:lysate or with 12D7:NY-ESO-1 presented NY-ESO-1-derived epitopes approximately equally well (Figure 2B).

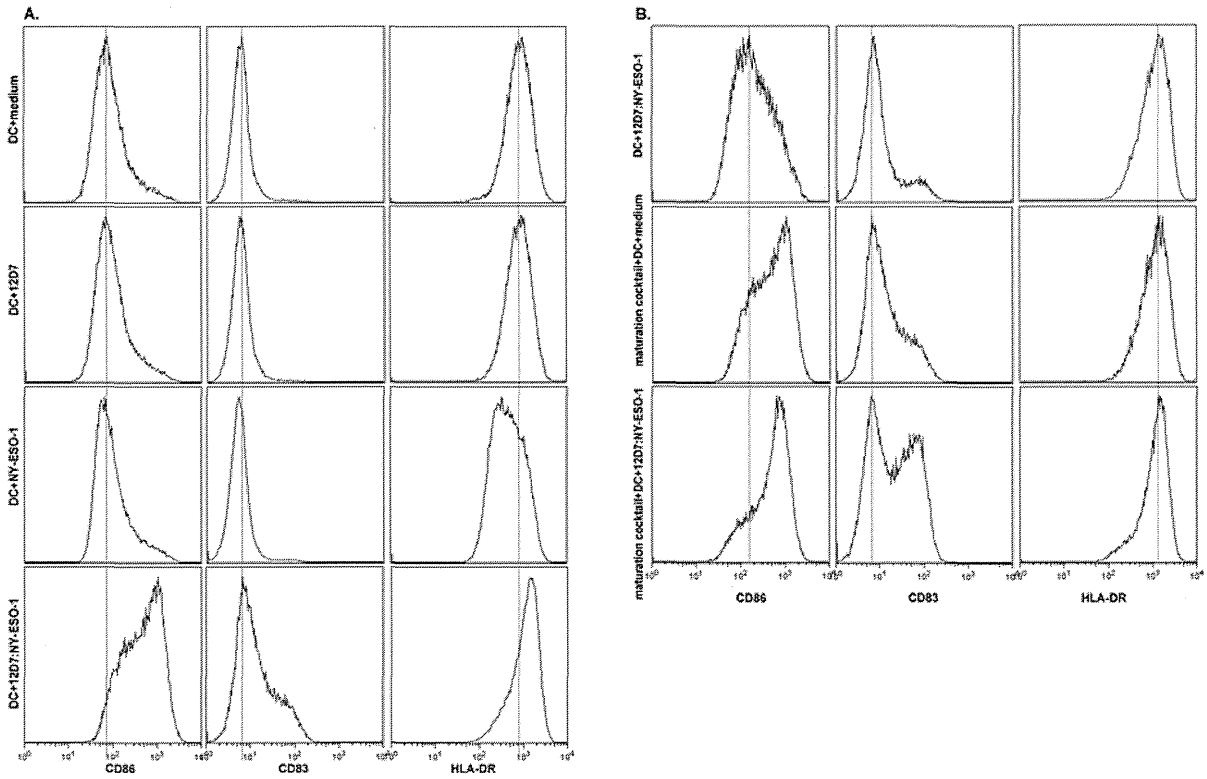
Because presentation of 12D7:NY-ESO-1 immune complexes seemed not to require deliberate DC maturation, we addressed whether the uptake of immune complexes, but not the uptake of uncomplexed protein, induced DC maturation *in vitro*. We therefore compared the expression of three surface molecules that are upregulated on mature DCs (CD83, CD86, and MHC class II) after incubation with media, 12D7, NY-ESO-1, or with 12D7:NY-ESO-1 immune complexes in the absence of maturation cocktail. We found that only immune complexes induced an upregulation of CD86, CD83, and MHC II (Figure 3A; left, middle, and right panels, respectively). We then compared the expression of CD83, CD86, and MHC II on DCs that were incubated with the maturation cocktail, with immune complexes, or with both, in order to determine the relative potency of immune complexes with respect to DC maturation. We found that immune complexes were almost as potent in inducing DC maturation as the classical maturation cocktail (sCD40L plus TNF-α) (Figure 3B). A combination of immune complexes plus maturation cocktail resulted in the most pronounced upregulation of CD86 and CD83 (Figure 3B; left and middle panels, respectively), whereas MHC II was not further upregulated compared to any of the two treatments alone (Figure 3B, right panels).

12D7 increases the therapeutic efficacy of chemotherapy in mice with NY-ESO-1+ tumors

To test the therapeutic efficacy of 12D7 *in vivo*, we injected 10^6 syngeneic, NY-ESO-1-transfected CT26 tumor cells s.c. in BALB/c mice. To induce release of intracellular NY-ESO-1, mice were treated with 75 mg/kg 5-FU when tumors reached a size of approximately 25 mm^2, which was typically around 2 weeks after injection of tumor cells. The treatment with 5-FU was repeated one week later and, in some groups, was combined with 100 μg 12D7 given systemically 2 d after each 5-FU injection. As can be seen from the growth curves, 5-FU has the expected therapeutic effect. Importantly, this was enhanced by 12D7 (Figure 4A). Treatment with 12D7 alone had no effect, presumably because the amount of spontaneously released antigen is not sufficient in this particular model. A compilation of end-point tumor sizes from 4 independent experiments shows a highly significant difference between mice treated with 5-FU plus 12D7, and mice treated with 5-FU alone (Figure 4B).

To investigate whether treatment with 5-FU plus 12D7 supported tumor-specific immunity, we injected mice with Brefeldin A 4 h before euthanasia, followed by staining for CD45.2, CD8, and intracellular IFN-γ. This way of analysis shows which cells actually are making IFN-γ *in vivo* and not which cells potentially can do this upon *in vitro* restimulation with peptide. This method obviously does not allow discrimination between single peptide specificities, but it is of higher biological relevance (25) particularly because we envisaged that DC activation, which we have shown to occur upon cross-presentation (Figure 3), may also support the presentation of other epitopes besides those derived from NY-ESO-1. Treatment with 5-FU plus 12D7 supported CD8+ and

Figure 3



Immune complexes induce maturation of monocyte-derived DCs *in vitro*. (A) CD14-derived DCs were incubated with media, 200 μg NY-ESO-1, 20 μg 12D7 or preformed immune complexes of 20 μg 12D7 + 200 μg NY-ESO-1, and were analyzed 36 h later for surface expression of CD86, CD83, or MHC II. (B) CD14-derived DCs were incubated with preformed immune complexes of 20 μg 12D7 + 200 μg NY-ESO-1, maturation cocktail (sCD40L + TNF-α) or with preformed immune complexes plus maturation cocktail, and were analyzed 36 h later for surface expression of CD86, CD83, or MHC II.

effector function in the tumor (Figure 4C). Treatment with 5-FU (Figure 4C) or 12D7 (data not shown) did not have this effect.

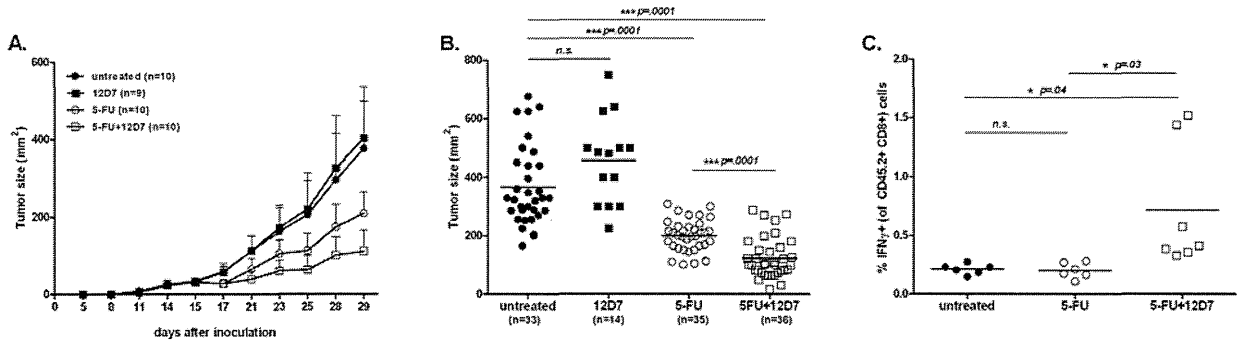
Discussion

We hypothesized that antibodies against intracellular, tumor-associated antigens support tumor-specific immunity when used in combination with a therapy that induces cell death such as chemo- or radiotherapy. We envisaged that such antibodies form immune complexes with the released tumor antigens. These immune complexes are subsequently taken up with higher efficiency compared to protein (fragments) by DCs (26), which then cross-present relevant epitopes to local CD8+, tumor-specific T cells. This presumed sequence of events may be of particular interest as evidence is accumulating that both chemo- and radiotherapy support tumor-specific immunity (27), and we therefore reasoned that additional stimulation of tumor-specific immunity could further improve the efficacy of these standard therapies.

For this purpose, we have cloned the first fully human mAbs to NY-ESO-1 using Epstein-Barr virus (EBV)-transformed B cells from a melanoma patient and subjected those to preclinical experiments to obtain proof of principle. We found that 12D7, a fully human IgG1 mAb specific for the immunogenic CT antigen NY-ESO-1, supported cross-presentation of NY-ESO-1 *in vitro* resulting in an approximate 15-fold increase of the number of responding CD8+ T cells. Of the other four NY-ESO-1-specific mAbs we generated here, 1D4 and 30D6 improved cross-presentation of NY-ESO-1 (data not shown), whereas 15B12 and 31E4 seemed not effective (data not shown). This difference may be explained by the difference in affinity, as 15B12 did not show binding to NY-ESO-1 by Biacore—although it did bind weakly to NY-ESO-1 in ELISA—and 31E4 had at least a 1-log lower affinity than 12D7, 1D4, and 30D6. At present, we have no reason to think that the epitope recognized by the mAb impacts on its ability to support cross-presentation. Our observation that 12D7:NY-ESO-1 immune complexes are considerably less efficient than peptide-loaded DCs in stimulating IFN-γ production illustrates that cross-presentation is a rather inefficient process, but underscores the therapeutic potential of antibodies against tumor-associated antigens.

It is well accepted now that activation of T cells *in vivo* crucially depends on antigen presentation by mature or activated DCs (14, 28). Many cues, including inflammation and infection but also endogenous signals, can induce DC maturation (29), and the lack of such signals in the tumor environment may be one reason why tumor-infiltrating T cells often have compromised functions (16, 30). Because the uptake of immune complexes was shown to result in DC maturation (19), we specifically addressed this issue here. We found that the *in vitro* uptake of immune complexes resulted in DC maturation that was comparable to sCD40L plus TNF-α, which is a classical maturation cocktail. Therefore, the use of mAbs against CT antigens may serve both purposes: DC activation and enhanced

Figure 4



A human, monoclonal anti-NY-ESO-1 antibody (12D7) increases the therapeutic efficacy of 5-FU chemotherapy in mice bearing NY-ESO-1+ syngeneic tumors. Female BALB/c mice were injected s.c. with 10^6 CT26/NY-ESO-1+ cells and treatment was started when tumors reached a surface of approximately 25 mm^2 (~ d13-15). (A) Mice received 75 mg/kg 5-FU i.p. at days 15 and 22 and/or 100 µg 12D7 i.p. on days 17 and 24. The results are shown as mean±SD. A representative experiment of 4 experiments is shown. (B) Compilation of 4 independent experiments, each symbol represents the tumor surface of an individual mouse at the end of the experiment (d 29). (C) Mice were injected i.p. 1 week after the last injection with 12D7 (d 29) with 250 µg Brefeldin A and were euthanized 4 h later. Processing of tumors and staining with antibodies for CD45.2, CD8 (surface), and IFN-γ (intracellular) was performed in the presence of 10 µg/mL Brefeldin A. Each symbol represents values from individual mice at the end of the experiment.

cross-presentation at the relevant anatomic location. This is not trivial, as systemic activation of DCs may not be without risk as systemic side effects such as the release of cytokines or autoimmunity may ensue (31, 32).

We found that 12D7 improved the efficacy of chemotherapy in a preclinical mouse model of transplanted, syngeneic NY-ESO-1-expressing tumors, thus supporting our concept. Further support comes from the fact that more CD8+ T cells infiltrate the tumor and that those cells have increased effector function. By itself, however, 12D7 had no therapeutic effect, suggesting that the amount of released tumor antigen is limiting without deliberate destruction of the tumor. Our *in vivo* experiments require the binding of human IgG to mouse Fcγ receptors (FcγR), which was previously described (33, 34). Improved efficacy of chemotherapy by the use of tumor-associated antigen-specific antibodies will presumably work for chemotherapies especially, which are not immunosuppressive or—even more important—promote immunogenic cell death (35).

We propose the concept of antibody-facilitated T cell induction in cancer (AFTIC) as a novel type of immunotherapy. AFTIC is based on the application of mAbs against tumor-associated antigens, including CT antigens, plus a treatment that promotes the local release of those antigens, such as chemo- or radiotherapy. The locally released antigens and the mAb form immune complexes, which facilitate the uptake and subsequent presentation of antigen-derived peptides by tumor-associated DCs. As the uptake of immune complexes induces concomitant maturation of DCs, AFTIC supports boosting as well as *de novo* activation of tumor-specific CD8+ T cells. Furthermore, administration of antibodies against a particular tumor-associated antigen may promote the presentation of the same antigen when administrated as a cancer vaccine, thereby improving the efficacy of immunotherapy. Alternatively, better antigen presentation of immune complexes and concomitant DC maturation may support the activity of adoptively transferred T cells provided they have the same antigen specificity as the therapeutic antibodies.

Abbreviations

CT, Cancer/Testis; DC, dendritic cell; mAb, monoclonal antibody

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Materials and methods

Patient material

Serum and peripheral blood was collected from cancer patients. All patients were admitted at the University Hospital Zürich and provided written informed consent in accordance with the Declaration of Helsinki. The local ethics committee approved the study.

Memory B cell culture

PBMC were incubated with anti-CD22 coupled to magnetic beads (Miltenyi Biotec), PE-conjugated anti-IgD, and APC-conjugated antibodies to IgM, CD3, CD8, and CD56 (Becton Dickinson). B cells were isolated by positive selection of CD22+ cells using a midi-MACS device and LS columns (Miltenyi Biotec), followed by sorting PE-APC- cells using a MoFlo cell sorter (Beckman Coulter). CD22+ IgD- IgM- memory B cells were incubated with 10% EBV-containing supernatant from B95-8 cells (from European Collection of Cell Cultures, ECACC) in the presence of 2.5 μg/mL CpG 2006 at 37°C for 4 h. Cells were seeded in 96-well U-bottom plates at 10 cells per well plus 3 x 10^4 irradiated allogeneic PBMCs in RPMI 1640 medium supplemented with 10% human serum, antibiotics, 10% supernatant from B95-8 cells, and 2.5 μg/mL CpG 2006. Supernatants were tested for NY-ESO-1-specific antibodies after 2 weeks by ELISA.

Single cell-RT-PCR

B cell cultures were harvested and single cells were deposited into a 96-well PCR plate (Applied Biosystems) using a MoFlo XDP cell sorter (Beckman Coulter). RT-PCR was performed using random hexamer primers for cDNA synthesis and specific primers to amplify the immunoglobulin variable and constant regions. Immunoglobulin heavy and light chain variable regions were amplified using a nested PCR approach as described (36). Primer-encoded amino acid sequences and J-C regions of the antibodies were corrected to represent the authentic amino acid sequence as it occurred in the patient in a subsequent step prior to antibody production.

Antibody production and purification

293-T human embryonic kidney cells were transfected with 25 kDa branched polyethylenimine (PEI, Polysciences, Warrington, PA) plus DNA plasmids (heavy and light chain in equal ratios) in a 1.3:1 ratio and were incubated for 15 min at room temperature. Following transfection, the cells were cultured in serum free Opti-MEM I + GlutaMAX-I (Invitrogen) supplemented with 10 U penicillin-streptomycin (Lonza, Switzerland). After 72 h supernatants were collected and IgG was purified on a protein A column (GE Healthcare, Sweden) using FPLC (GE Healthcare, Sweden).

Biacore analysis

Antibody binding kinetics with NY-ESO-1 proteins derived from *E. coli* (LICR New York Branch) and HEK293 cells (OriGene Technologies, Inc.) were determined by Biacore technology (model Biacore 2000; Biacore AB) using CM5 sensor