学位論文の関連論文の研究背景及び要旨

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〔題名〕

Combined adjuvants of poly(I:C) plus LAG-3-Ig improve anti-tumor effects of tumor-specific T cells preventing their exhaustion（poly(I:C)とLAG-3-Igのアジュバント併用が腫瘍特異的T細胞疲弊を阻害し抗腫瘍効果を改善する）

[研究背景]　がんワクチン療法は内因性の免疫応答を刺激し、形成したがんを治療することを目的とする。1990年代に腫瘍関連抗原（TAA）が同定され、MHC抗原分子エピトープやTAA蛋白質などをワクチンの抗原として利用できるようになった。その結果、様々ながん種に対して多くのがんワクチン臨床治験が実施されたが、大規模臨床治験の最終段階で全生存や無病生存期間における有意な改善は認められなかった。臨床効果が不十分である主な理由として少なくとも二つの可能性が考えられる。一つ目はTAA特異的なT細胞が疲弊または不応答状態となっており、腫瘍細胞に対する免疫応答が効率的に誘導できないこと、二つ目はリンパ組織でTAA特異的なT細胞が活性化しても、腫瘍微小環境において免疫抑制メカニズムで無力化されることである。効果的ながんワクチン療法の開発には、これらの問題を克服する必要がある。

上記の問題点を解決する戦略の一つはアジュバントの最適化である。アジュバントは抗原に対する免疫応答を増強するワクチン成分として使用される。Montanide (IFA)やQS21、Monophosphoryl lipid Aなど様々なアジュバントが臨床治験で使用されており、その生物学的効果はTAAの運搬の改善と抗腫瘍免疫応答の増強を目的としている。前者はワクチン製剤を安定化しin vivoで投与したTAAのワクチン効果延長と最適な生体内分布をもたらす一方、後者は主にパターン認識受容体やToll様受容体の一部を通して自然免疫を刺激することで二次的にTAA特異的なT細胞応答を誘導する。アジュバント製剤を改良することで、現在のがんワクチン療法の問題点を克服し、優れた臨床結果を達成できる可能性がある。

[目的]　本研究では、がんワクチンによる免疫誘導効果を最適化しうるアジュバントの開発を目指す。そのために、免疫増強活性を有するpoly(I:C)とLAG-3-Igを併用する新規アジュバントを考案した。これを利用したがんワクチン治療の有効性とその抗腫瘍メカニズムの解析を目的とする。

[方法]　DBA/2マウスにP815マストサイトーマを$5x10^5$個皮下接種して腫瘍形成を確認した後、腫瘍接種後7日目に腫瘍抗原であるP1Aに特異的なTCR遺伝子導入T細胞を$2x10^5$個輸注した。さらに腫瘍接種後8、15日目にアジュバント存在下でP1Aペプチドワクチンを50μg皮下接種した。アジュバントとしてIFA 50μl、poly(I:C) 50μg、LAG-3-Ig 1μg、またはpoly(I:C)とLAG-3-Igの併用を投与し、腫瘍増殖とマウス生存率により抗腫瘍効果を比較検討した。抗腫瘍効果メカニズムの解析として、腫瘍が完全退縮し100日以上生存

したマウスにP815を再接種した場合のP1A特異的メモリー反応の有無、腫瘍接種後21日目に採取した腫瘍組織の免疫組織染色、およびマウスの腫瘍所属リンパ節におけるP1A特異的T細胞の増殖とサイトカイン産生、免疫疲弊マーカーの発現を検討した。

[結果]　アジュバントとしてpoly(I:C)とLAG-3-Igを併用した群では、すべてのマウスで腫瘍が完全退縮した。一方、IFA群では腫瘍接種後50日目までに全マウスが腫瘍増大により死亡した。また、poly(I:C)単独群、LAG-3-Ig単独群ではIFA群に比べ腫瘍増殖の遅延を認めたものの、poly(I:C)単独群で腫瘍が退縮した1匹を除き、他のマウスはすべて腫瘍増大により死亡した。これらの結果は、poly(I:C)とLAG-3-Igの併用がP1Aペプチドワクチンの抗腫瘍効果を増強しうる強力なアジュバント剤であることを示唆した。

また、同マウスモデルを用いて腫瘍接種後21日目に採取した腫瘍組織の病理組織学的検討を行った。H&E染色とCD4とCD8分子の免疫組織染色を実施したところ、poly(I:C)とLAG-3-Igの併用群にて腫瘍組織のネクローシスやCD4陽性、CD8陽性T細胞の浸潤増強が認められた。これらの結果からpoly(I:C)とLAG-3-Igの併用は腫瘍微小環境において腫瘍反応性T細胞応答を誘導していることが示唆された。

次に、併用アジュバントによりP815が完全退縮し100日以上生存したマウスに、P815の再接種、もしくは同系の腫瘍細胞株であるがP815と抗原性の異なるL1210の皮下接種をおこなった。その結果、L1210接種では腫瘍増大が認められたが、P815は増大せず完全拒絶されたことから、腫瘍特異的メモリー反応の誘導が示唆された。

抗腫瘍効果のメカニズムを解析するために、上記マウスモデルの腫瘍接種後21日目に腫瘍所属リンパ節細胞を採取し、P815存在下で3日間培養して抗原刺激し、細胞増殖とサイトカイン産生を比較検討した。その結果、併用群にて有意に高い細胞増殖とTh1型サイトカイン産生の増強が認められた。さらに免疫疲弊マーカーであるPD-1、LAG-3、TIGIT、BTLAの発現レベルをFACSにて検討したところ、P1A特異的T細胞においてこれらの分子の発現低下が認められた。以上の結果から、poly(I:C)とLAG-3-Igのアジュバント併用投与によるがんペプチドワクチンの治療増強効果は腫瘍抗原特異的T細胞の疲弊状態の解除と関連があることが示唆された。

[結論]　poly(I:C)とLAG-3-Igの併用は腫瘍特異的T細胞の疲弊状態を解除し、細胞増殖とTh1型サイトカイン産生を増強することで、優れた抗腫瘍効果を誘導しうる有用性の高いアジュバントであることが示された。

Original article:

Combined adjuvants of poly(I:C) plus LAG-3-Ig improve anti-tumor effects of tumor-specific T cells preventing their exhaustion

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Abstract

Therapeutic cancer vaccines are designed to treat cancer by boosting the endogenous immune system to fight against the cancer. In the development of clinically-effective cancer vaccine, one of the most practical objectives is to identify adjuvants which are capable of optimizing the vaccine effects. In this study, we explored a potential of poly(I:C) and LAG-3-Ig as adjuvants for P1A tumor antigen peptide vaccine in the pre-established P815 mouse tumor model with a transfer of tumor-specific T cells. Whereas a usage of poly(I:C) or LAG-3-Ig as a signal adjuvant induced a slight enhancement of P1A vaccine effects compared to IFA, combined administration of poly(I:C) plus LAG-3-Ig remarkably potentiated anti-tumor effects, leading to complete rejection of pre-established tumor and long-term survival of mice. The potent adjuvant effects of poly(I:C) plus LAG-3-Ig were associated with an enhanced infiltration of T cells in the tumor tissues, and an increased proliferation and Th1-type cytokine production of tumor-reactive T cells. Importantly, the combined adjuvant of poly(I:C) plus LAG-3-Ig downregulated expressions of PD-1, LAG-3, and TIGIT on P1A-specific T cells, indicating prevention of T cell exhaustion. Taken together, current study demonstrated that the combined adjuvants of poly(I:C) plus LAG-3-Ig with tumor peptide vaccine induce profound anti-tumor effects by activating tumor-specific T cells.

Key words

Cancer vaccine, adjuvants, poly(I:C), LAG-3, T cell exhaustion

Abbreviations

BTLA	B- and T-lymphocyte attenuator
BV	Brilliant Violet
DAPI	4',6-Diamidino-2-phenylindole
PD-1	Programmed cell death 1
Poly(I:C)	Polyinosinic-polycytidylic acid
TIGIT	T cell immunoglobulin and ITIM domain

Introduction

Therapeutic cancer vaccine is a strategy to stimulate endogenous immune responses against tumor cells, so as to aiming at a cure of established cancers. This approach has been strengthened by the identification of tumor-associated antigens (TAA) in the 1990s, which enabled us to take advantage of short peptides of MHC class I/II epitopes, long peptides spanning multiple epitopes, or TAA protein itself, as antigens (Ag) for vaccine (1-3). Accordingly, a large number of clinical trials of cancer vaccine have been examined in various types of cancers worldwide (1, 4, 5). Contrary to great expectation and enthusiasm, the vast majority of late-stage clinical trials of cancer vaccine resulted in a failure to meet primary or secondary endpoints of showing significant improvement in overall survival or progression-free/disease-free survival (5-7). Only exceptional case so far is sipuleucel-T, a cell-based vaccine against patients with metastatic hormone-refractory prostate cancer, which was approved by FDA in 2010 (8). Insufficient clinical efficacy in the majority of therapeutic cancer vaccines could be attributed to two potential reasons. First, TAA-specific T cells are rendered unresponsive due to exhaustion or anergy in patients with advanced cancer, being an obstacle to elicit productive immune responses against tumor cells (6, 9). Second, even TAA-specific T cells are activated in lymphoid organs, they will be rendered

deactivated when exposed to various immunosuppressive mechanisms in the tumor microenvironment (6, 10). The approaches of therapeutic cancer vaccines need to be improved so as to overcome these issues.

A potential strategy to address above issues in cancer vaccine is an optimization of adjuvants. An adjuvant is any substance used as a vaccine component which boosts immune responses against Ag. Various adjuvants including montanide (IFA; incomplete Freund's adjuvant), QS21 (saponin), monophosphoryl lipid A (MPL), liposome, cytokines, etc., have been used in clinical trials of cancer vaccine (1, 4, 5). Biological effects of adjuvants can be categorized into two major modes of action, i.e. an improved delivery of TAA and an acceleration of anti-tumor immune responses. The former effect is based on stabilization of vaccine formulations which leads to an extended presence and preferred bio-distribution of vaccinated TAA in vivo. The latter effect is predominantly mediated by a stimulation of innate immune cells via signaling into pattern recognition receptors (PRR), particularly toll-like receptors (TLR), which leads to indirect activation of TAA-specific T cell responses. In spite of these potentials, the current formulations of adjuvant need to be further improved, so as to render therapeutic cancer vaccine capable of achieving sufficient clinical outcomes.

In this study, we investigated combination of poly(I:C) plus LAG-3-Ig as a novel

adjuvant for therapeutic tumor peptide vaccine. Poly(I:C), a synthetic double-stranded RNA, binds TLR3, melanoma differentiation-associated protein 5 (MDA-5) and retinoic acid-inducible gene I (RIG-I), induces production of type I interferon (IFN) and IL-12, and upregulates cross-priming of DC, thus leading to activation of TAA-specific T cells (11, 12). On the other hand, LAG-3-Ig, a soluble recombinant protein of lymphocyte activation gene-3 (LAG-3) extracellular domain fused with human IgG Fc region, has been reported to competitively attenuate LAG-3 inhibitory signal in T cells as well as to stimulate dendritic cells (DC) and monocytes by interaction with MHC class II (13-15). Whereas previous reports have demonstrated a potential of poly(I:C) and LAG-3-Ig as an adjuvant separately used for cancer vaccine (11, 16-21), no studies have investigated whether combination of these two reagents could strengthen their effects. We here explore poly(I:C) plus LAG-3-Ig as a novel combination of adjuvants, which can synergistically enhance therapeutic efficacy of cancer vaccine to stimulate tumor-specific T cell responses.

Material and methods

Mice and cell lines

In all experiments, 6-10 weeks-old female DBA/2 mice, purchased from Japan SLC, Inc. (Shizuoka, Japan), were used. P1A-specific T cell receptor (TCR)-transgenic mice (22) were originally generated and kindly provided by Dr. Yang Liu (The Children's Research Institute, Washington DC), and backcrossed with DBA/2 mice at least 10 generations in our animal facility. All mice were maintained under specific pathogen-free conditions in the animal facility at the Yamaguchi University. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Yamaguchi University.

P815 mastocytoma and L1210 lymphocytic leukemia, both syngeneic to DBA/2 mice, were purchased from ATCC and maintained in vitro with RPMI 1640 culture medium (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gemini bio products, West Sacramento, CA), 1% penicillin-streptomycin (WAKO, Osaka, Japan), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 50 mM 2-mercaptoethanol (Thermo Fisher Scientific, MA).

In vivo therapeutic model of pre-established tumor

DBA/2 mice were inoculated subcutaneously (s.c.) with 5 x 10^5 P815 tumor cells in the lateral flank on day 0. On day 7, spleen cells from P1A-specific TCR-transgenic mice which contained 2 x 10^5 P1A-specific T cells identified as Vα8.3-positive cells by flow cytometry analysis were transferred intravenously (i.v.) into the mice. On day 8 and 15, the mice were injected s.c. with 50 μg P1A peptide (LPYLGWLVF: Sigma-Aldrich, St. Louis, MO) mixed with following adjuvants; 50 μl Incomplete Freund's Adjuvant (IFA: Sigma-Aldrich), 50 μg poly(I:C) (InvivoGen, San Diego, CA), 1 μg LAG-3-Ig (Adipogen, San Diego, CA), or 50 μg poly(I:C) plus 1 μg LAG-3-Ig. Tumor growth was measured periodically with digital calipers and tumor volume was calculated by the following formula; tumor volume (mm^3) = (short diameter)^2 x long diameter/2. Survival of the mice was also observed.

The mice which had completely rejected tumor and survived over 100 days via P1A peptide vaccine with adjuvants were re-challenged s.c. with 5 x 10^5 P815 cells in the left lateral flank and 5 x 10^5 L1210 cells in the right lateral flank. As a control, naïve DBA/2 mice were inoculated s.c. with P815 and L1210 in the same method. Tumor growth and survival of mice were monitored as above.

Histopathological and immunofluorescence analysis of tumor tissue

DBA/2 mice were inoculated with P815 tumor on day 0, injected with P1A-specific T cells on day 7, and then treated with P1A peptide vaccine with adjuvants on day 8, as described above. On day 14, tumors were excised from the mice and divided into two pieces by razor blade. One piece was immersed and fixed in 10% formalin solution, and used for H&E staining conducted by Biopathology Institute Co. Ltd (Oita, Japan). The other piece was embedded in OCT compound (Sakura Finetek, Tokyo, Japan) to generate frozen sections of tumor.

Immunofluorescence staining was performed by using 5 μm thick sections cut from the frozen tumor tissue. Tissue sections were placed on a slide and fixed with methanol at -20°C for 10 min. The slides were then washed with PBS, followed by blocking with 3% BSA in PBS at RT for 30 min. Tissue sections were stained with anti-mouse CD4 Ab (rat IgG2b) and anti-mouse CD8α Ab (rat IgG2a) at 4°C for overnight (both Abs were purchased from eBioscience, San Diego, CA). The slides were then washed with PBS, followed by staining with Alexa Fluor 488-conjugated mouse anti-rat IgG2a Ab and Alexa Fluor 647-conjugated mouse anti-rat IgG2b Ab at RT for 60 min (both Abs were purchased from Abcam, Cambridge, MA). Finally, the slides were washed with PBS and mounted with ProLong Gold Antifade Reagent with DAPI (Thermo Fisher Scientific, Waltham, MA). Observation of the slides was performed by using BZ-X710

fluorescent microscope (KEYENCE, Osaka, Japan).

Cell proliferation and cytokine assay

DBA/2 mice were inoculated with P815 tumor on day 0, injected with P1A-specific T cells on day 7, and then treated with P1A peptide vaccine with adjuvants on day 8 and 15, as described above. On day 21, tumor-draining inguinal and axillary lymph nodes (LN) were harvested and processed to single cell suspension. LN cells (1.5×10^5 cells/well) were co-cultured with 100 Gy-irradiated P815 tumor cells (4×10^4 cells/well) in tissue-culture 96-well flat-bottom plates (Thermo Fisher Scientific, MA). Proliferative activity of the cells was assessed by ^{3}H-thymidine incorporation during the last 10 hrs of 3-days culture. Determination of the incorporated radioactive counts was measured by a TopCount NXT (Perkin Elmer, Waltham, MA).

To assess a cytokine production from tumor-reactive T cells, supernatants from the above co-culture of tumor-draining LN cells and irradiated P815 cells were harvested after 3 days. The concentrations of various cytokines were measured by Bio-Plex Pro Mouse 23-plex assay kits according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA).

FACS analysis

DBA/2 mice were inoculated with P815 tumor, and treated with P1A-specific T cell transfer and P1A peptide vaccine with adjuvants, as described above. On day 21, tumor-draining LN cells were harvested stained with BV421-conjugated anti-CD4 Ab, APC-conjugated anti-CD8 Ab, and FITC-conjugated anti-Vα8.3 Ab. To assess T cell exhaustion markers, the cells were further stained with PE-conjugated anti-PD-1 Ab, PE-conjugated anti-LAG-3 Ab, PE-conjugated anti-TIGIT Ab, and PE-conjugated anti-BTLA Ab. Flow cytometric data were acquired by BD LSRFortessa X-20 cell analyzer (BD Biosciences, San Jose, CA), and the data were analyzed by FlowJo Cytometry Analysis (Tree Star, Ashland, OR). Abs used for FACS analysis were purchased from eBioscience or BioLegend (San Diego, CA).

Statistical analysis

Unpaired, two-tailed Student's *t*-test was used for parametric data such as cytokine and proliferation data, while log-rank test was used for mouse survival data. The results are expressed as the mean±SD. Differences were considered to be significant at P values less than 0.05.

Results

Eradication of pre-established P815 tumor by P1A peptide vaccine together with combined adjuvant of poly(I:C) plus LAG-3-Ig

In order to evaluate an efficacy of poly(I:C) and LAG-3-Ig as immunological adjuvants in tumor vaccine, we utilized an in vivo murine tumor model of pre-established P815 mastocytoma. DBA/2 mice, syngeneic to P815 tumor, were inoculated with P815 tumor cells s.c. on day 0. After 7 days, when the tumor mass reached approximately 6 mm diameter, the mice were injected i.v. with P1A-specific TCR-transgenic T cells, followed by vaccination of P1A peptide together with IFA, poly(I:C), LAG-3-Ig, or both poly(I:C) and LAG-3-Ig on day 8 and 15. While P1A is a dominant TAA in P815 tumor (23), it has been reported that vaccination of P1A peptide alone is insufficient to induce regression of pre-established P815 tumor (24). When P1A peptide vaccine with IFA adjuvant was given, all the mice suffered from outgrowth of tumor and died by day 50 (**Fig. 1A, 1B**). Although vaccination of P1A peptide together with poly(I:C) or LAG-3-Ig adjuvant delayed the growth of P815 tumor, almost all the mice were eventually killed by the tumor. In sharp contrast, when the mice were treated with P1A peptide vaccine together with both poly(I:C) and LAG-3-Ig, pre-established P815 tumor completely regressed and all of the mice survived indefinitely. These results indicate

that combination of poly(I:C) plus LAG-3-Ig works as a highly potent adjuvant which enhances anti-tumor therapeutic effects of P1A peptide vaccine .

Increased infiltration of T lymphocytes in tumor tissue by P1A peptide vaccine together with poly(I:C) plus LAG-3-Ig adjuvant

We also performed histopathological examination of tumor tissues in the mice treated with P1A peptide vaccine together with adjuvants. The mice were inoculated s.c. with P815 tumor on day 0, subsequently injected i.v. with P1A-specific TCR-transgenic T cells on day 7, and then treated with P1A peptide vaccine with IFA, poly(I:C), LAG-3-Ig, or both poly(I:C) and LAG-3-Ig on day 8. On day 14, tumor tissues were harvested and subjected to H&E staining as well as immunohistochemical staining of CD4 and CD8 to evaluate T cell infiltration. In the tissues from the mice treated with IFA as an adjuvant, massive growth of tumor cells with a lack of T cell infiltration was observed (**Fig. 2A, 2B**). In the mice treated with either poly(I:C) or LAG-3-Ig, necrotic area was observed in a part of tumor tissues, while only a slight infiltration of T cells was detected. In the mice treated with combined adjuvant of poly(I:C) and LAG-3-Ig, majority of tumor tissues underwent necrotic changes, and an evident infiltration of CD4 and CD8-positive T cells adjacent to the necrotic area was observed. In addition,

enhanced expressions of MHC class I and class II were observed in tumor tissues from the mice treated with both poly(I:C) and LAG-3-Ig (**Fig. S1**). These findings confirm that the combined adjuvant of poly(I:C) plus LAG-3-Ig together with peptide vaccine greatly enhances tumor-reactive T cell responses and MHC expressions, probably due to IFN-γ produced by infiltrating T cells, at the tumor microenvironment.

Development of tumor-specific memory responses in the mice treated with P1A peptide vaccine together with poly(I:C) plus LAG-3-Ig adjuvant

DBA/2 mice which were treated with P1A peptide vaccine together with combined adjuvant of poly(I:C) plus LAG-3-Ig completely eradicated pre-established P815 and survived over 100 days (Figure 1). In order to explore the development of P815-specific long-term T cell memory, the mice which had rejected tumor were re-challenged with P815 or L1210, a tumor cell line syngeneic to DBA/2 mice but irrelevant to P815 in terms of antigenicity. The tumor-rejected mice were resistant to the second challenge of P815 but not to the primary challenge of L1210 (**Fig. 3**). As control, inoculation of same numbers of P815 and L1210 into naïve DBA/2 mice led to outgrowth of both tumors. These results thus indicate that therapeutic effects of tumor peptide vaccine together with combined poly(I:C) plus LAG-3-Ig adjuvant induces tumor-specific long-term

memory responses.

Enhanced proliferation and Th1-type cytokine production in the mice treated with P1A peptide vaccine together with poly(I:C) plus LAG-3-Ig adjuvant

In order to explore underlying mechanism of anti-tumor effects induced by Poly I:C and LAG-3-Ig adjuvants with peptide vaccine, proliferative activity and cytokine production of tumor-reactive T cells were assessed. The mice were inoculated s.c. with P815 tumor on day 0, injected i.v. with P1A-specific TCR-transgenic T cells on day 7, and then treated with P1A peptide vaccine with IFA, poly(I:C), LAG-3-Ig, or both poly(I:C) and LAG-3-Ig on day 8 and 15. On day 21, tumor-regional lymph node (LN) cells were harvested and co-cultured with irradiated P815 tumor cells. Proliferative responses of LN cells were significantly enhanced when the mice were treated with combined adjuvant of poly(I:C) plus LAG-3-Ig, compared to either one of them or IFA (**Fig. 4A**). Similarly, production of IFN-γ and GM-CSF was increased by the combined adjuvant of poly(I:C) plus LAG-3-Ig to the levels significantly higher than the other groups (**Fig. 4B**). On the other hand, increase of IL-4 and IL-5 production was triggered by poly(I:C) and LAG-3, respectively, and the combination of poly(I:C) plus LAG-3 led to only a modest increase compared to poly(I:C) or LAG-3-Ig alone. As to IL-17, its production

induced by the combination of poly(I:C) plus LAG-3-Ig was lower than that by poly(I:C) alone, but still higher than IFA. These results suggest that efficient anti-tumor effects mediated by the combined adjuvant of poly(I:C) plus LAG-3-Ig were associated with enhanced T cell proliferation and cytokine production, characterized as a preferential upregulation of Th1-type, but not Th2 or Th17-type, responses.

To further confirm therapeutic effects of combined adjuvants of poly(I:C) plus LAG-3-Ig, we additionally assessed proliferative activity and IFN-γ production of tumor-reactive T cells without an adoptive transfer of tumor-specific TCR-transgenic T cells. C57BL/6 mice were inoculated s.c. with B16-F10 tumor on day 0, treated with gp100 peptide vaccine with IFA, poly(I:C), LAG-3-Ig, or both poly(I:C) and LAG-3-Ig on day 8. On day 14, tumor-regional lymph node (LN) cells were harvested and co-cultured with gp100 peptide. Proliferative responses of LN cells and IFN-γ secretion were significantly enhanced when the mice were treated with combined adjuvants of poly(I:C) plus LAG-3-Ig (**Fig. S2**). This result confirm an efficacy of combined adjuvants of poly(I:C) plus LAG-3-Ig in a distinct tumor model without an adoptive transfer of tumor-specific T cells.

Prevention of tumor-reactive T cell exhaustion by the combined adjuvant of

poly(I:C) plus LAG-3-Ig

It has been demonstrated that tumor-reactive T cells undergo exhausted status in the hosts suffering from progressive tumors (25). Moreover, successful immunotherapies are often associated with prevention and/or reversal of T cell exhaustion (26). Therefore, we investigated whether injections of poly(I:C) and LAG-3-Ig as adjuvants could influence exhausted phenotypes of tumor-reactive T cells. The mice were inoculated s.c. with P815 tumor on day 0, injected i.v. with P1A-specific TCR-transgenic T cells on day 7, and then treated with P1A peptide vaccine with IFA, poly(I:C), LAG-3-Ig, or both poly(I:C) and LAG-3-Ig on day 8 and 15. On day 21, tumor-regional LN cells were harvested and analyzed for the expression of exhaustion markers, including PD-1, LAG-3, TIGIT, and BTLA, on the P1A-specific cytotoxic T lymphocytes (CTL), which were identified as Vα8.3-positie, CD8-potitive cells (22). It was found that approximately 40-50% of P1A-specific CTL expressed PD-1 in the mice treated with IFA as an adjuvant (**Fig. 5**). While administration of poly(I:C) or LAG-3-Ig as a single adjuvant inhibited PD-1 expression to some extent compared to IFA, combination of poly(I:C) plus LAG-3-Ig showed synergistic effects to remarkably downregulate PD-1 level on P1A-specific CTL. Similarly, expressions of LAG-3 and TIGIT were most strikingly inhibited by the combined adjuvant of poly(I:C) plus LAG-3-Ig. On the other

hand, almost no changes in BTLA expression levels were observed by the combined adjuvant of poly(I:C) plus LAG-3-Ig, compared to IFA. Since it was reported that CD4-positive, Vα8.3-positie T cells from P1A-TCR transgenic mice also recognize P1A epitope and exert cytotoxic functions against P815 tumor (27), we further examined exhaustion markers on CD4/Vα8.3-double positive T cells. Similarly to P1A-specific CTL, expressions of PD-1, LAG-3, and TIGIT, but not BTLA, on CD4-positive P1A-specific T cells were downregulated by the combined adjuvant of poly(I:C) plus LAG-3-Ig (**Fig. S3**). Taken together, these results indicate that anti-tumor effects induced by peptide vaccine together with combined adjuvant of poly(I:C) plus LAG-3-Ig are associated with prevention of tumor Ag-specific T cell exhaustion.

Discussion

The current study has demonstrated that administration of combined adjuvant of poly(I:C) plus LAG-3-Ig profoundly enhances anti-tumor responses induced by tumor peptide vaccine and leads to complete regression of pre-established tumor in association with long-term immunological memory. Furthermore, mechanistic analyses revealed activation of Th1-type responses and prevention of exhausted phenotype in tumor-specific T cells by this treatment. To the best of our knowledge, our study is the first to report a great advantage of combined adjuvants of poly(I:C) plus LAG-3-Ig for therapeutic cancer vaccine.

Immunologically, there are multiple mechanisms how adjuvants augment anti-tumor immune responses. Poly(I:C) activates DC to produce type I IFN and IL-12, both of which mediate stimulatory effects on anti-tumor T cell responses (28-30). Poly(I:C) also enhances cross-presentation of exogenous Ag by DC which is necessary for priming CTL specific to TAA epitope in the context of MHC class I (31, 32). In addition, a potential role of poly(I:C) on NK cells and tumor cells have been reported (33, 34). On the other hand, LAG-3-Ig interferes with inhibitory LAG-3 signal in T cells and prevents them from undergoing exhausted status (18). In addition, LAG-3-Ig binds MHC class II with a higher affinity than CD4, and induces maturation and activation of

DC which leads to upregulated expressions of CD80, CD83, and CD86 (14, 35). Thus, combination of poly(I:C) and LAG-3-Ig could orchestrate multiple, non-overlapping mechanisms of immune stimulation, which account for profound synergy in therapeutic effects by anti-tumor vaccine. Fundamental mode of action in poly(I:C) plus LAG-3-Ig adjuvant would be expected as follows; DC acquire enhanced APC functions and efficiently present TAA to tumor-specific T cells under the cytokine milieu preferential for Th1-type responses. Subsequently, tumor-specific T cells are activated and efficiently eliminate tumor cells while preventing T cell exhaustion due to resistance to immune inhibitory mechanisms. Lysis of tumor cells is followed by cross-presentation of TAA, which triggers epitope spreading to activate a broad repertoire of tumor-specific T cells, leading to more efficient elimination of tumors.

Recent advances in immune checkpoint blockade therapy, particularly anti-PD-1 Ab, demonstrated remarkable clinical benefits to prolong overall survival and/or progress-free survival in various types of advanced cancers (36). In this regard, it should be noted that combined adjuvant of poly(I:C) and LAG-3-Ig is capable of downregulating multiple immune checkpoint molecules on tumor-specific T cells, including PD-1, LAG-3, and TIGIT. It was reported that combined immunotherapies of poly(I:C) with PD-L1/PD-1 blockade in the presence or absence of cancer vaccine

induced potent anti-tumor effects (37, 38). In addition, efficacy of immunotherapies of anti-PD-1 Ab combined with cancer vaccine or other checkpoint blockade including anti-LAG-3 Ab has been demonstrated (39). Thus, downregulation of multiple immune checkpoint molecules by poly(I:C) plus LAG-3-Ig could be a pivotal event accounting for powerful anti-tumor effects of this vaccine therapy.

Induction of tumor-specific long-term memory is one of the most important features of cancer immunotherapy, which could protect patients from tumor recurrence. It has been reported that poly(I:C) as an adjuvant of tumor peptide vaccine induces memory CTL responses, in which CD4-positive helper T cells play a supportive role (40, 41). Regarding the effects on $CD4^+$ T cells, our study took advantage of P1A-sepcific TCR transgenic T cells, in which $CD4^+$ T cells are capable of responding to P1A peptide in a MHC class I-restricted manner as reported by a previous study (27) and confirmed by our experiment (data not shown). Thus, we consider that the enhanced proliferation and cytokine productions of T cells by the combined adjuvant of poly(I:C) plus LAG-3-Ig might be mediated by $CD4^+$ T cells in some extent, whereas detailed functions of $CD4^+$ T cells in our model need to be elucidated by further experiments. In addition, LAG-3 signal is known to regulate the quantity of memory T cells in vivo (42). Thus, it is plausible that combined adjuvant of poly(I:C) plus LAG-3-Ig can induce long-term

immune memory and protection from tumor re-challenge as shown in this study. LAG-3 signal was also reported to play a crucial role in suppressive functions of regulatory T cells (Treg) (43). On the other hand, immunological effects of poly(I:C) seems to irrelevant to Treg number and functions (17, 44). In the current study, we could not detect any changes of Treg number in the mice treated with combined adjuvant of poly(I:C) plus LAG-3-Ig, compared to other groups (data not shown), suggesting a negligible contribution of Treg to the effects in our approach.

Selection of appropriate and optimized adjuvants is a crucial issue which could govern a clinical success of cancer vaccine therapy. Although IFA has been applied to various clinical trials as a common adjuvant of cancer vaccine, recent study revealed that TAA-specific T cells are sequestered in the vaccine site and undergo dysfunction and deletion by usage of IFA (45). Besides IFA, poly(I:C) is currently used in over 20 active clinical trials of cancer vaccine, mainly in glioblastoma, melanoma, and ovarian cancer, by itself or combination with other adjuvants (11). Although most of trials are still in early-stage and thus give no conclusive statement yet, currently available information indicate potent immunological effects and promising clinical responses by poly(I:C) (16, 17). LAG-3-Ig has been applied to clinical trials targeting renal cell carcinoma, melanoma, pancreatic cancer, and breast cancer, as an adjuvant of cancer

vaccine or combination with chemotherapy (18-21). Thus, both poly(I:C) and LAG-3-Ig are readily available as GMP-grade compounds. In addition, their safety profile has been exhibited by multiple phase I clinical trials. Based on the findings in this study, we are planning to implement clinical trials using combined adjuvant of poly(I:C) plus LAG-3-Ig together with tumor peptide vaccine in near future.

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Disclosure

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Figure legends

Figure 1. Eradication of pre-established P815 tumor by P1A peptide vaccine together with combined adjuvant of poly(I:C) plus LAG-3-Ig

DBA/2 mice were inoculated s.c. with P815 tumor cells on day 0. After 7 days, mice were injected i.v. with P1A-specific TCR-transgenic T cells, followed by vaccination of P1A peptide together with IFA, poly(I:C), LAG-3-Ig, or both poly(I:C) and LAG-3-Ig on day 8 and 15. (A) The growth of P815 tumor was measured periodically. Each line represents tumor size of individual mouse, and a dagger symbol (†) indicates a death of mouse. (B) Survival of mice is shown. Each symbol represents distinct adjuvants as follows. □: IFA, ○: poly(I:C), ■: LAG-3-Ig, ●: poly(I:C) plus LAG-3-Ig. The survival of mice treated with the combined adjuvant of poly(I:C) and LAG-3-Ig was significantly longer than the other groups ($P<0.05$). Representative data of two independent experiments are shown.

Figure 2. Histopathological analyses of P815 tumor tissues in the mice treated with various adjuvants along with P1A peptide vaccine

DBA/2 mice were inoculated s.c. with P815 tumor cells on day 0. After 7 days, mice were injected i.v. with P1A-specific TCR-transgenic T cells, followed by vaccination of

P1A peptide together with IFA, poly(I:C), LAG-3-Ig, or both poly(I:C) and LAG-3-Ig on day 8. On day 14, tumors were surgically resected and subjected to histopathological analyses. (A) Representative images of H&E staining are shown (x200). Insets indicate high-power magnification of each image (x400). (B) Representative images of immunohistochemistry staining by anti-CD4 Ab (red), anti-CD8 Ab (green), and DAPI (blue) were shown (x200).

Figure 3. Induction of P815-specific memory response by the treatment with P1A peptide vaccine together with combined adjuvant of poly(I:C) plus LAG-3-Ig

DBA/2 mice were inoculated s.c. with P815 tumor cells on day 0. After 7 days, mice were injected i.v. with P1A-specific TCR-transgenic T cells, followed by vaccination of P1A peptide together with poly(I:C) and LAG-3-Ig on day 8 and 15. Over 100 days later, the tumor-rejected mice (○) were re-challenged s.c. with P815 and L1210 cells at the left and right lateral flank, respectively. As a control, naïve DBA/2 mice (●) were also inoculated s.c. with P815 and L1210 in a same manner. The growth of tumors was measured periodically and is shown as the mean±SD.

Figure 4. Proliferation and cytokine production of tumor-draining LN cells in the

mice treated with P1A peptide vaccine together with combined adjuvant of poly(I:C) plus LAG-3-Ig

DBA/2 mice were inoculated s.c. with P815 tumor cells on day 0. After 7 days, mice were injected i.v. with P1A-specific TCR-transgenic T cells, followed by vaccination of P1A peptide together with IFA, poly(I:C), LAG-3-Ig, or both poly(I:C) and LAG-3-Ig on day 8 and 15. On day 21, tumor-draining LN cells were harvested and cultured with 100Gy-irradiated P815. (A) Proliferative activity of the tumor-draining LN cells during the last 10 hrs of 3-days culture was assessed by ^{3}H-thymidine incorporation. (B) After 3 days, the culture supernatants were harvested and the concentrations of cytokines were determined. Representative data of two independent experiments are shown as the mean±SD of triplicate samples. *; $P<0.05$, **; $P<0.01$, N.S.; not significant.

Figure 5. Expression of exhaustion markers on tumor-reactive CTL in the mice treated with P1A peptide vaccine together with combined adjuvant of poly(I:C) plus LAG-3-Ig

DBA/2 mice were inoculated s.c. with P815 tumor cells on day 0. After 7 days, mice were injected i.v. with P1A-specific TCR-transgenic T cells, followed by vaccination of P1A peptide together with IFA, poly(I:C), LAG-3-Ig, or both poly(I:C) and LAG-3-Ig

on day 8 and 15. On day 21, tumor-draining LN cells were harvested and analyzed on the expressions of exhaustion markers including PD-1, LAG-3, TIGIT, and BTLA, along with CD8 and Vα8.3 by flow cytometer. Expressions of exhaustion markers on P1A-specific TCR-transgenic CTL, gated as CD8/Vα8.3-double positive cells, were shown. The numbers indicate percentages of exhaustion marker-positive cells within the P1A-specific CTL population. Representative data of two independent experiments are shown.

Supporting Information

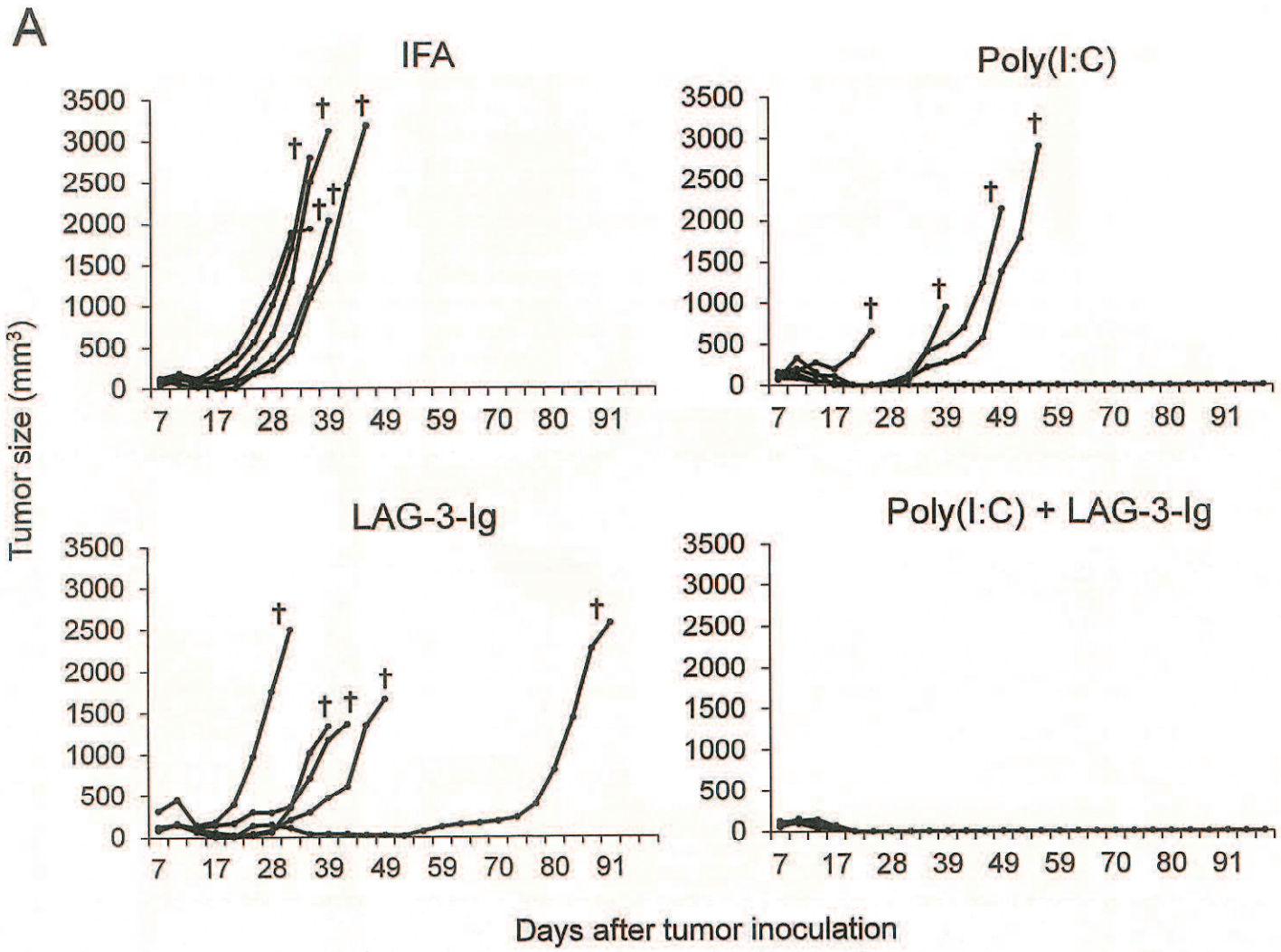
Additional supporting information is shown in the online version of this article.

Fig. S1. Expression of MHC class I/II in tumor tissues

Fig. S2. T cell responses induced by gp100 peptide vaccine with adjuvants

Fig. S3. Expression of exhaustion markers on tumor-reactive $CD4^+$ T cells

Figure 1


A
IFA
Poly(I:C)
LAG-3-Ig
Poly(I:C) + LAG-3-Ig
Tumor size (mm³)
3500
3000
2500
2000
1500
1000
500
0
7 17 28 39 49 59 70 80 91
Days after tumor inoculation

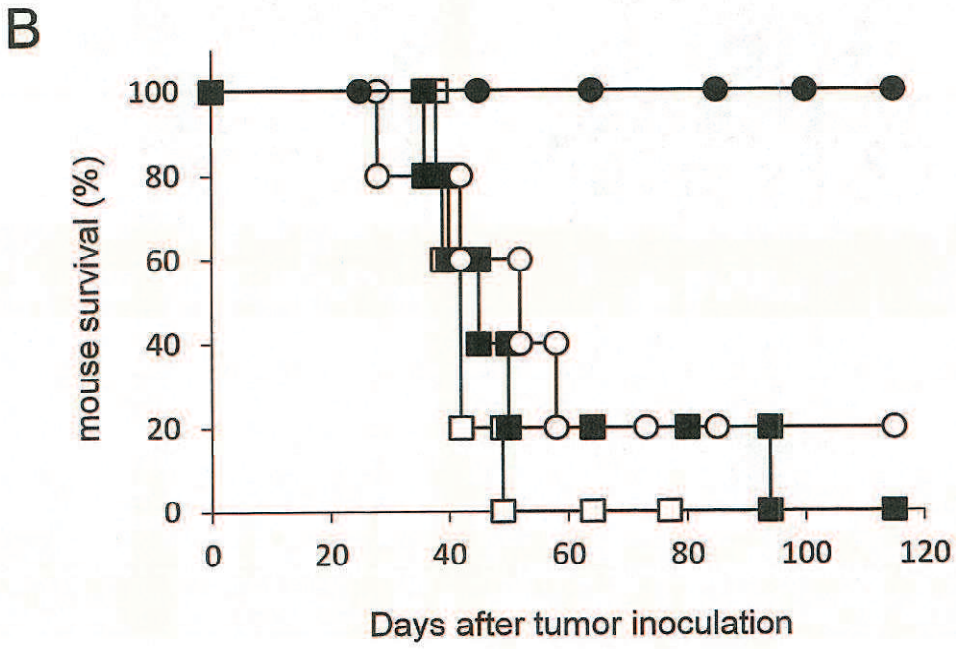

B
mouse survival (%)
100
80
60
40
20
0
0 20 40 60 80 100 120
Days after tumor inoculation

Figure 2

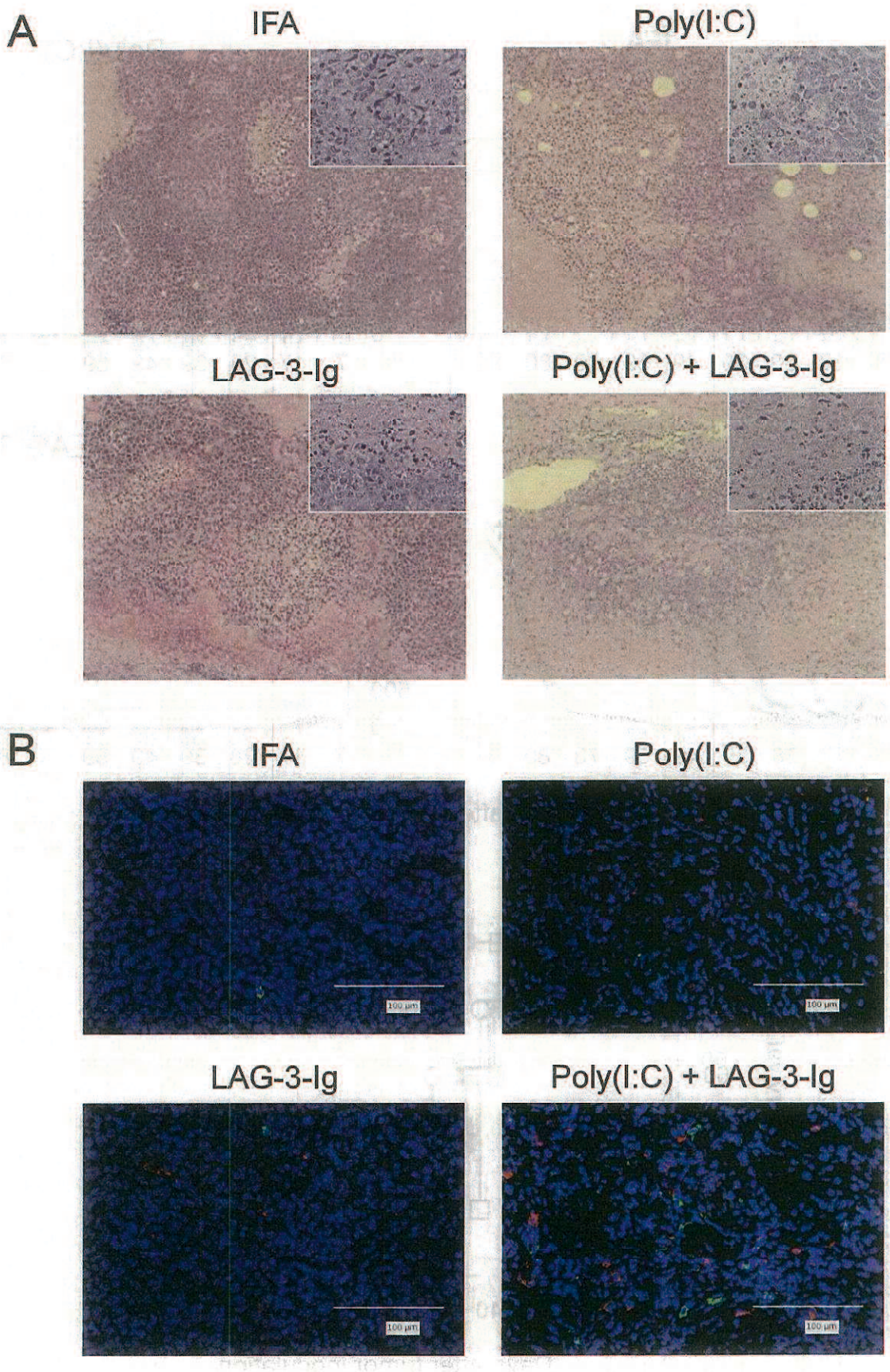

A
IFA
Poly(I:C)
LAG-3-Ig
Poly(I:C) + LAG-3-Ig
B
IFA
Poly(I:C)
LAG-3-Ig
Poly(I:C) + LAG-3-Ig
100 µm
100 µm
100 µm
100 µm

Figure 3

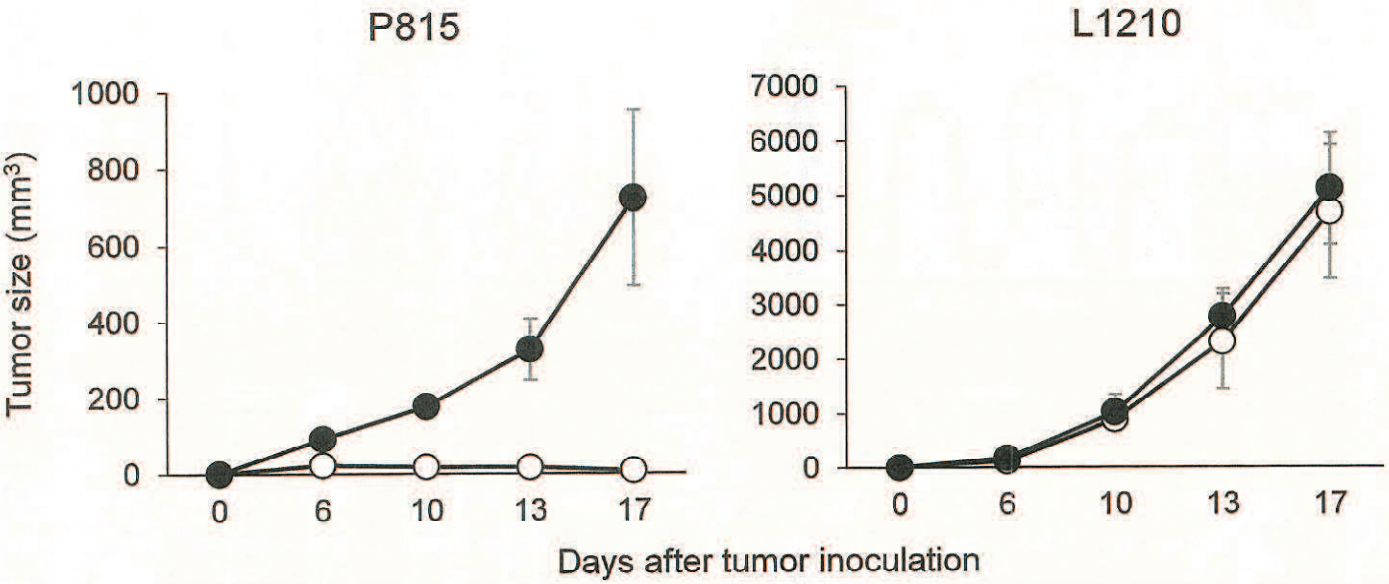

P815
L1210
Tumor size (mm³)
1000
800
600
400
200
0
7000
6000
5000
4000
3000
2000
1000
0
0
6
10
13
17
Days after tumor inoculation

Figure 4

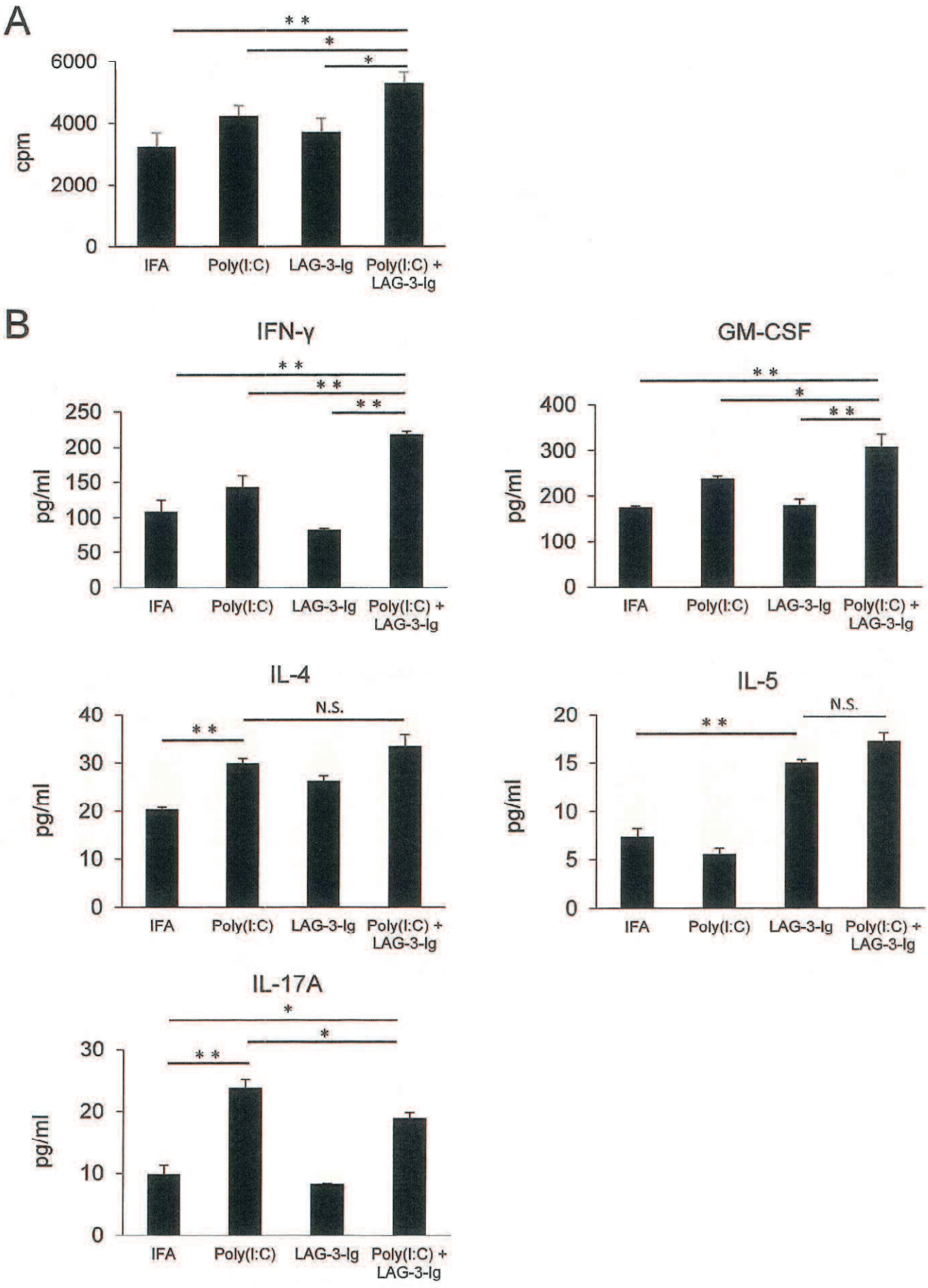
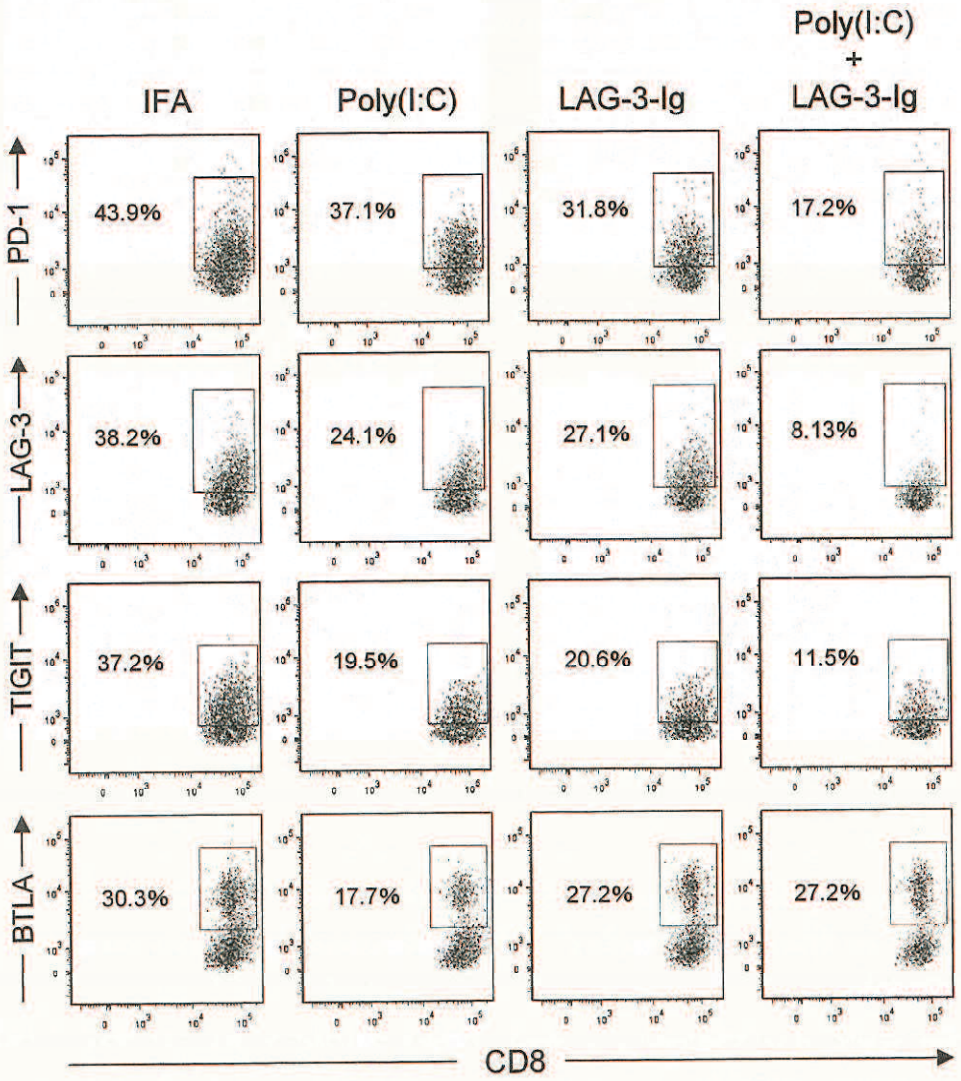
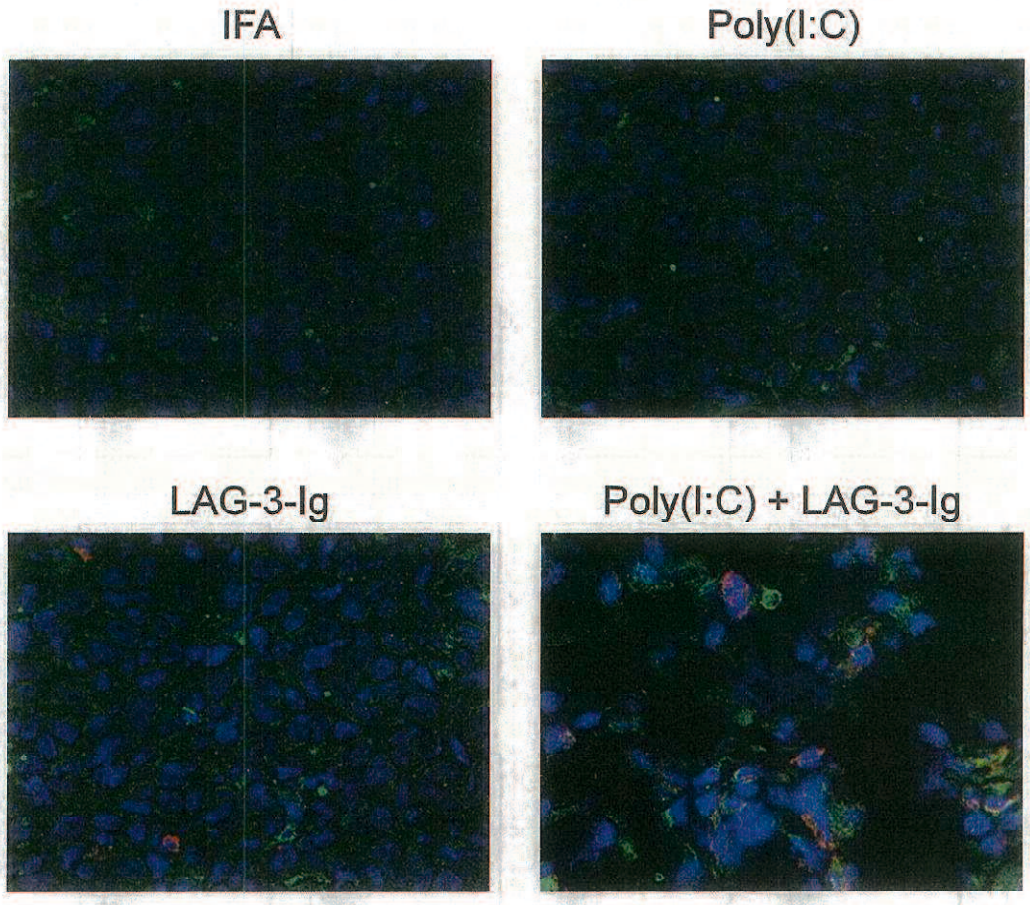

A
cpm
6000
4000
2000
0
* *
*
*
IFA
Poly(I:C)
LAG-3-Ig
Poly(I:C) + LAG-3-Ig
B
IFN-γ
pg/ml
250
200
150
100
50
0
GM-CSF
400
300
200
100
IL-4
40
30
20
10
N.S.
IL-5
15
5
IL-17A

Figure 5


IFA
Poly(I:C)
LAG-3-Ig
Poly(I:C) + LAG-3-Ig
PD-1
LAG-3
TIGIT
BTLA
CD8
43.9%
37.1%
31.8%
17.2%
38.2%
24.1%
27.1%
8.13%
37.2%
19.5%
20.6%
11.5%
30.3%
17.7%
27.2%
27.2%

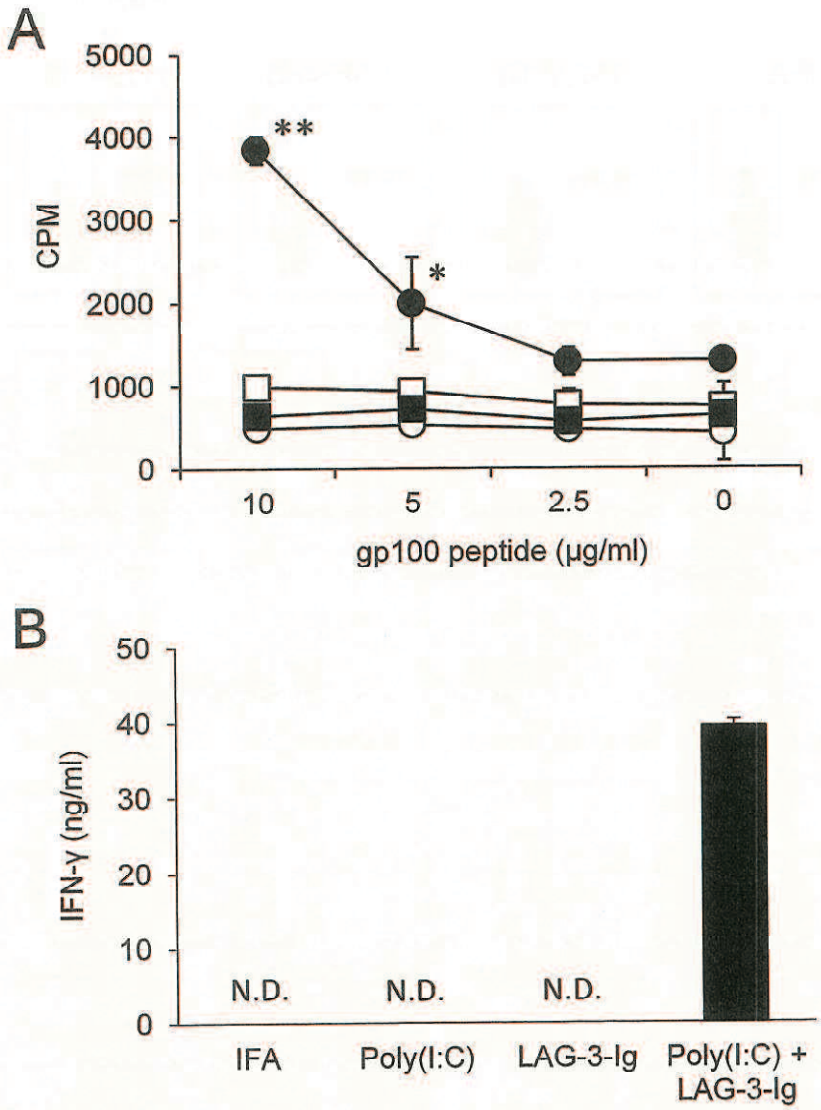
Supplementary Figure 1



Supplementary Figure 1. Expressions of MHC class I and class II in P815 tumor tissues in the mice treated with various adjuvants along with P1A peptide vaccine

DBA/2 mice were inoculated s.c. with P815 tumor cells on day 0. After 7 days, mice were injected i.v. with P1A-specific TCR-transgenic T cells, followed by vaccination of P1A peptide together with IFA, poly(I:C), LAG-3-Ig, or both poly(I:C) and LAG-3-Ig on day 8. On day 14, tumors were surgically resected and subjected to histopathological analyses. Representative images of immunohistochemistry staining by anti-mouse H-2L^{d} Ab (green), anti-I-A/I-E^{d} Ab (red), and DAPI (blue) were shown (x400).

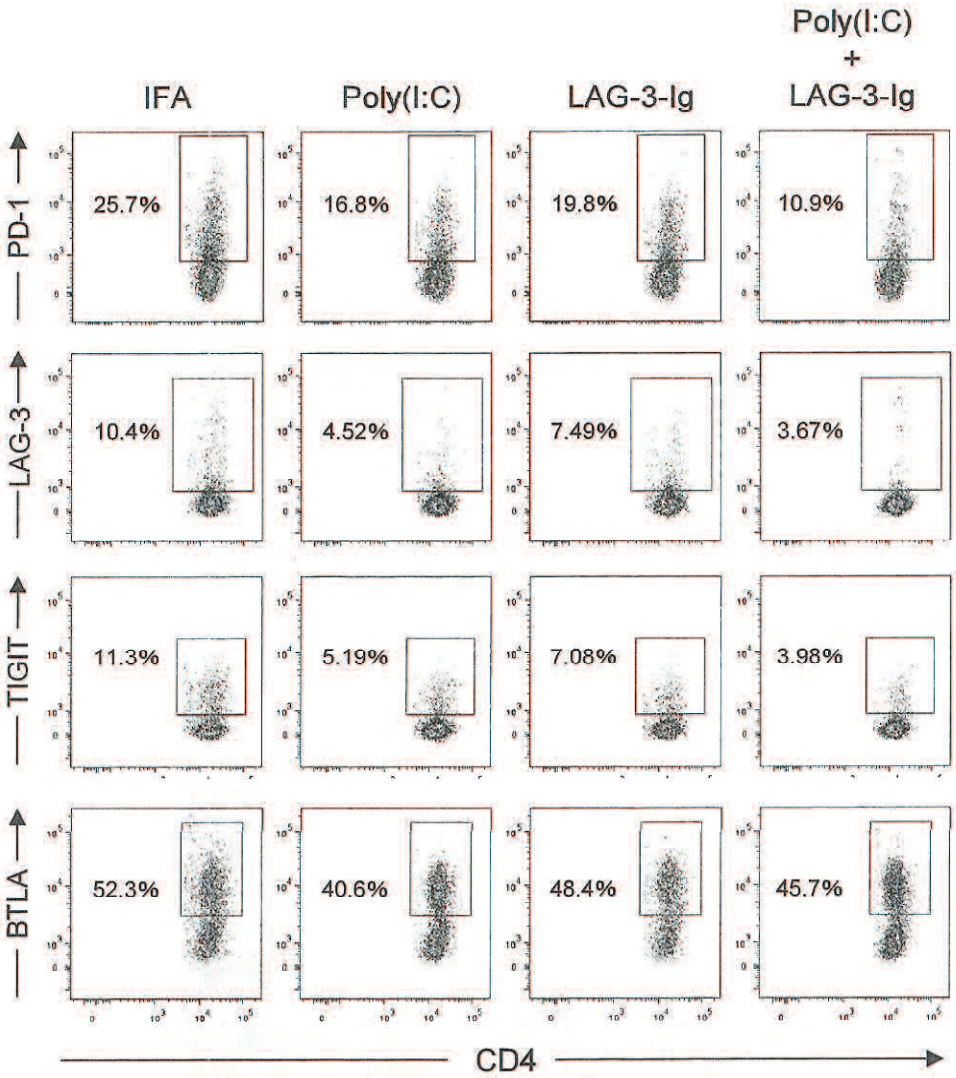
Supplementary Figure 2



Supplementary Figure 2. Proliferation and cytokine production of tumor-draining LN cells in the mice treated with gp100 peptide vaccine together with combined adjuvant of poly(I:C) plus LAG-3-Ig

C57BL/6 mice were inoculated s.c. with B16-F10 tumor cells on day 0. On day 8, the mice received vaccination of gp100 peptide together with IFA (open square), poly(I:C) (open circle), LAG-3-Ig (closed square), or both poly(I:C) and LAG-3-Ig (closed circle). On day 14, tumor-draining LN cells were harvested and cultured in the presence of gp100 peptide. (A) Proliferative activity of the tumor-draining LN cells during the last 10 hrs of 3-days culture was assessed by ^{3}H-thymidine incorporation. (B) After 3 days, the culture supernatants were harvested and the concentrations of IFN-γ were determined. Data are shown as the mean +/- SD of triplicate samples. *; $P<0.05$, **; $P<0.01$, N.D.; not detectable.

Supplementary Figure 3



Supplementary Figure 3. Expression of exhaustion markers on tumor-reactive CD4⁺ T cells in the mice treated with P1A peptide vaccine together with combined adjuvant of poly(I:C) plus LAG-3-Ig

DBA/2 mice were inoculated s.c. with P815 tumor cells on day 0. After 7 days, mice were injected i.v. with P1A-specific TCR-transgenic T cells, followed by vaccination of P1A peptide together with IFA, poly(I:C), LAG-3-Ig, or both poly(I:C) and LAG-3-Ig on day 8 and 15. On day 21, tumor-draining LN cells were harvested and analyzed on the expressions of exhaustion markers including PD-1, LAG-3, TIGIT, and BTLA, along with CD4 and Va8.3 by flow cytometer. Expressions of exhaustion markers on P1A-specific TCR-transgenic CD4⁺ T cells, gated as CD4/Va8.3-double positive cells, were shown. The numbers indicate percentages of exhaustion marker-positive cells within the P1A-specific CD4⁺ T cell population. Representative data of two independent experiments are shown.