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CD4-positive diffuse large B-cell lymphoma: A variant with aggressive clinical potential

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Abstract

CD4 expression is rare in diffuse large B-cell lymphoma (DLBCL), with 4 previously reported cases. Its significance is uncertain. We report five patients with $CD4^+$ DLBCL and one $CD4^+$ primary mediastinal large B-cell lymphoma. Cases were identified by searching the electronic database of the department; each was reviewed. Average age was 56 years. Neoplastic cells expressed CD20 (5/6 tested cases). BCL2/BCL6 expression were seen in 3/3 tested cases, suggesting a germinal center origin. Additionally, expression of T-cell antigens CD2 and CD5 was noted in 2/2 and CD7 in 1/1 tested case. CD3 was negative in all. Lymph nodes were commonly involved (67%). Patients received chemotherapy +/- radiation (6/6) and bone marrow transplant (2/6). Average survival was 44.2 mo. CD4 expression in DLBCL raises questions of lineage commitment. $CD4^+$ DLBCL is rare; care should be exercised not to diagnose these as T-cell lymphomas. A subset behaves aggressively.

Key words: $CD4^+$; Diffuse large B-cell lymphoma; T-cell lymphoma; Lineage infidelity

Core tip: Aberrant expression of T-cell antigens including CD4 in ALK-negative diffuse large B-cell lymphoma (DLBCL) is a rare phenomenon that raises interesting biological and diagnostic considerations. With regards to our series of DLBCLs expressing CD4, it appears that at least a subset may behave aggressively based on our data.

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INTRODUCTION

Most cases of diffuse large B-cell lymphoma (DLBCL) show expression of B-cell antigens CD19, CD20, CD22, and CD79a. Variable expression of CD10, BCL-6, IRF4/MUM1, FOXP1, and less commonly CD5, is also observed. Surface/cytoplasmic light chain expression is usually present[1].

The expression of T-cell associated antigens is not seen in benign lymphoid proliferations and is uncommon in B-cell non-Hodgkin lymphoma (B-NHL), most frequently occurring in the setting of chronic lymphocytic leukemia/small lymphocytic lymphoma[2]. In a series of 101 B-NHL, CD2, CD5, and CD7 expression was seen in only one quarter of cases[3]. CD8 (2%-3% of cases) expression has also been documented[4]. CD3 and CD4 expression, however, are exceedingly rare in DLBCL with only 4 cases of the latter reported in the world literature to the best of our knowledge[5-7]. Herein, we report a series of six cases of $CD4^+$ large B-cell lymphoma (LBCL) identified from our institution and review the pertinent literature to determine the incidence and possible mechanisms of aberrant T-cell antigen expression in B-NHL.

LITERATURE

Permission for the study was granted by the Washington University (WU) Human Studies Committee. Consecutive cases diagnosed within a 21-year period in patients 0-99 years old were identified by searching the Copath database of the Division of Anatomic Pathology at Barnes Jewish Hospital/WU (WU/BJC) using the terms "large B-cell lymphoma" and "CD4" in any field. Each hit was then individually reviewed to identify cases of DLBCL with concomitant CD4 expression or aberrant expression of other T-cell antigens by immunohistochemistry or flow cytometry.

Clinical data were acquired from WU/BJC Clinical Desktop, CoPath, and Touchwork databases, and by clinician interview. Mortality data were also procured from the Social Security Death Index.

RESULTS

Histologic and clinical features for all six cases are detailed in Table 1. Patients ranged in age from 22-79 years (average 54 years), and included 3 men and 3 women. Due to the retrospective nature of the study information about extent of disease at presentation was limited to three patients, and ranged from relatively limited disease in patients 1 and 2 to more extensive involvement in patient 5. Lymph node biopsies were obtained from the cervical or supraclavicular regions in 4 patients. In patient 1 tissue from the left maxillary sinus was biopsied, and in patient 6 the skin of the right leg was sampled.

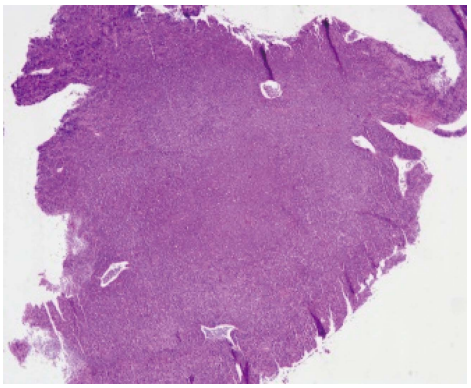

Figure 1 Diffuse effacement of underlying tissue architecture by neoplastic cells in left maxillary sinus biopsy, patient 1 (hematoxylin and eosin, original magnification × 400).

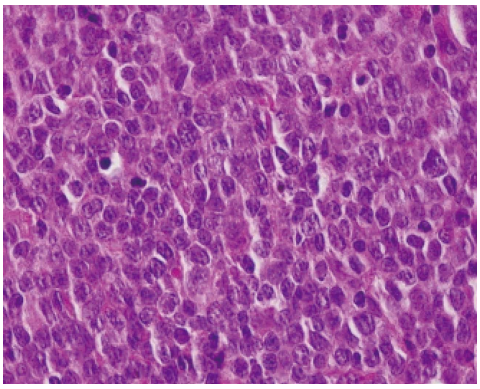

Figure 2 Large malignant cells with increased nuclear:cytoplasmic ratio, multiple nucleoli, irregular and nuclear contours arranged in sheets, case 1 (hematoxylin and eosin, original magnification × 1000).

Grossly, the tumors were noted to have a soft, grey/gelatinous appearance in two cases, and in one of these cases it was mistaken as a myxoma initially. In terms of immunophenotype, the neoplastic cells usually expressed CD45 (4/4 tested cases), CD20 (5/6 tested cases), CD19, and PAX5. BCL2, BCL6, and CD10 expression were also seen implying a germinal center origin (Figures 1-3). BCL2 and BCL6 expression were seen in 3/3 tested cases. CD4 was expressed in the vast majority of cells in 5/6 cases, and was positive in approximately 1/2 of the large cells in the biopsy from patient 4 (Figure 3). All cases were tested for CD3 expression by immunohistochemistry and were negative. With the exception of patient 4, the malignant cells in all patients were positive for CD20 immunohistochemistry. A monoclonal rearrangement of IgH by polymerase chain reaction (PCR) established the malignant lineage of the cells from patient 4. In addition to CD4, aberrant expression of T-cell antigens CD2 and CD5 was noted in two cases each and CD7 in one case. No TCR rearrangements were found in one tested case.

Five patients were diagnosed with DLBCL, including

Table 1 Summary of histologic and clinical features of ALK-negative, CD4-positive diffuse large B-cell lymphomas

Case no.	Age/sex	Diagnosis	Immunophenotype	Molecular findings	Gross description	Treatment	Clinical outcome	Site	Stage
1	73/M	DLBCL	CD20, BCL-2, CD10, CD5, CD43, BCL-6, and CD4		Soft, gelatinous appearing mass	R-CHOP × 4	Complete remission at 4 mo follow-up; free of disease at 6 mo follow-up	Left maxillary sinus	I-E
2	22/F	PMLBCL	CD45, CD20, CD79a, with variable expression of CD2, CD4, CD30, CD23, BCL-6 and BCL-2			12 cycles of R-VACOP-B; 2 cycles of R-ESHAP mediastinal radiation (40-50 Gy); matched, unrelated donor stem cell transplant with Bu/Cy conditioning; salvage chemotherapy with GND; SGN-35	Dead; 14 mo survival from disease discovery; Progressive disease	Supraclavicular lymph node	IIE-X-B
3	79/F	DLBCL and FL(3a)	CD45 (focal), CD20, CD3, CD21 (focal), Bcl-2, Pax-5, subset expression of CD2, CD4, CD5, CD7, CD8 Bcl-6, MUM1, and CD10			R-CHOP	Dead; 6 mo survival from disease diagnosis	Left neck lymph node	
4	67/M	CLL/SLL with transformation to LBCL	CD45, CD30, EMA, CD4 (subset), and CD43, rare weak CD2	46, XY[18]; clonal IGH rearranged; IGVH unmutated; TCR gamma rearrangement negative		Fludarabine and cytoxan × 3; fludarabine, Rituxan, and mitoxantrone × 2; R-CHOP × 4; R-CHOP; BEAM and auto transplant	Dead; 15 mo survival; Progressive disease	Left subclavicular lymph node	
5	26/M	B-cell large cell lymphoma	CD20, CD30 (weak), CD4 (subset)			R-CHOP × 5, field radiotherapy	No evidence of relapse at 72 mo; lost to follow up	Left posterior cervical lymph node	IV-A-E
6	55/F	Malignant lymphoma, diffuse cleaved large cell type, with B-cell differentiation	CD20, MB-2, CD4, BCL2, and CD43 PCNA				Lost to follow-up	Right leg skin	

CLL/SLL: Chronic lymphocytic leukemia/small lymphocytic lymphoma; CD: Cluster of differentiation; DLBCL: Diffuse large B-cell lymphoma; F: Female; M: Male; PMLBCL: Primary mediastinal large B-cell lymphoma; CHOP: Rituximab, cyclophosphamide, doxorubicin hydrochloride, vincristine sulfate; R-ESHAP: Rituximab, etoposide, methylprednisolone, high-dose cytarabine, and cisplatin; VACOP: Vinblastine, adriamycin, cyclophosphamide, etoposide, prednisone, bleomycin; GND: Gemcitabine, vinorelbine, and doxorubicin; SGN-35: Brentuximab vedotin; BEAM: Carmustine, etoposide, cytarabine, and melphalan; FL: Follicular lymphoma.

one (patient 4) in which the DLBCL represented a Richter transformation of a prior chronic lymphocytic leukemia/small lymphocytic lymphoma and one (patient 3) with DLBCL and associated grade 3a follicular lymphoma. Patient 4 presented with primary mediastinal large B-cell lymphoma.

Clinical follow-up was available for 5/6 patients, who were all treated with multiagent chemotherapy. Two patients received stem cell transplants. Two patients had an initial response until lost to further follow-up at 6 and 72 mo, respectively, and 3 died of progressive disease between 6-15 mo after original diagnosis. Average survival was 44.2 mo overall, and 11.7 mo for those who died.

CONCLUSION

Herein, we report the largest series of ALK-negative CD4-positive DLBCL. Five cases were DLBCL, NOS and one was an example of PMLBCL. In our cohort (*n*

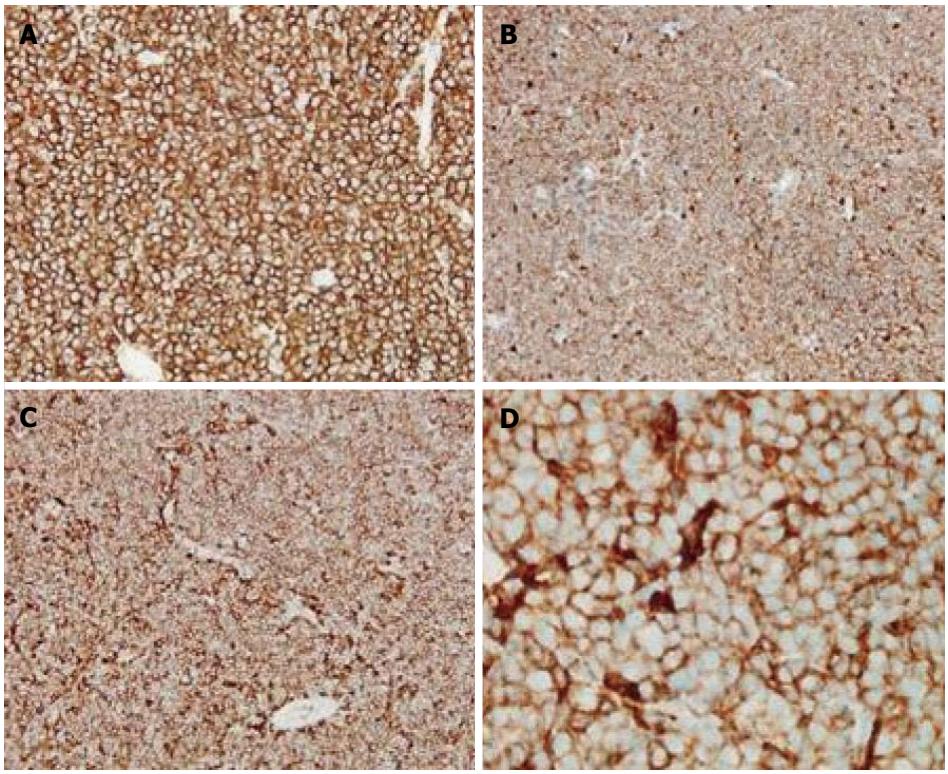

Figure 3 Immunohistochemistry results (clockwise). A: CD20 (original magnification × 200); B: CD5 (original magnification × 200); C: CD4 (original magnification × 200); D: CD4 (original magnification × 1000).

= 6), the average patient age was 56 years (range: 22-79). There was no sex predilection (3 males, 3 females). Lymph nodes were the most common site of involvement (67%). All patients received multi-agent chemotherapy, and two patients underwent stem cell transplantation. Average overall survival (n = 6) was 44.2 mo. For those who died (n = 3), average survival was only 11.7 mo.

The aberrant expression of T-cell associated antigens is not seen in benign lymphoid proliferations and is rather uncommon in B-NHL[7,8]. Nonetheless, aberrant expression of CD5, CD2, CD3, CD4, CD7, CD8, and CD45RO (UCHL-1) have all been reported in B-cell lymphomas. Most commonly, aberrant expression of T-cell antigens is seen in CLL and mantle cell lymphoma. Overall, the reported frequency of T-cell antigen expression in B-NHL ranges from 19% to 25%[8]. CD4 expression is unusual in B-NHL. It has been reported in plasmablastic lymphoma (3 cases), DLBCL associated with chronic inflammation/ pyothorax-associated lymphoma (1 case), and CLL (1 case)[9]. Interestingly, CD4 positivity is frequently seen in ALK+ DLBCL with one series citing a frequency of 64% (14/22 cases) in these rather uncommon neoplasms[10]. In DLBCL, NOS, CD4 expression is particularly rare with only 4 cases reported in the world literature to the best of our knowledge[6,8,11].

Review of the literature shows four prior reports or ALK-negative, CD4-positive DLBCL (Table 2). The earliest case was identified by Olack *et al*[8] who described a case of DLBCL that was positive for CD19, surface kappa light chain, CD4, and CD7 by flow cytometry. The patient was an 81-year-old man with moderate lymphadenopathy but no organomegaly or bone marrow involvement. He had stage Ⅲ disease and was lost to follow up after 13 mo of stable disease.

The next patient was an 82-year-old man who was found to have left cervical and left inguinal lymph node swelling. Biopsy of an involved lymph node showed DLBCL. Dual staining confirmed that the cells of interest were positive for both CD4 and CD19. Complete remission was achieved after five cycles of rituximab, cyclophosphamide, doxorubicin hydrochloride, vincristine sulfate (R-CHOP)[11].

2010, Arrondini *et al*[6] reported 2 cases of CD4+ DLBCL occurring in the small bowel. The first of these was a 55-year-old woman who presented with lymphoma entrapping the last part of the ileum, pancreas, and omentum. Molecular studies showed a clonal IgH rearrangement and no BCL2 rearrangement. The patient received dose-escalated (Mega) CHOP but two weeks later required a laparotomy for resection of 60 cm of ulcerated and perforated small bowel. The patient received 5 more cycles of R-MegaCHOP and achieved complete remission.

The second case reported by Arrondini *et al*[6] involved a 73-year-old man who presented with DLBCL involving the ileum. A staging bone marrow biopsy was negative. The patient was treated with adjuvant chemotherapy. Two years later, he presented with an enlargement of the pancreatic head and a mass involving the right

Table 2 Summary of ALK-negative, CD4-positive diffuse large B-cell lymphomas reported in the literature

Case no.	Age/sex	Diagnosis	Immunophenotype	Molecular findings	Gross description	Treatment	Clinical outcome	Site	Stage	Ref.
1	81/F	DLBCL	CD19, CD4, CD7				Lost to follow-up after 13 mo of stable disease	N/A	III	[2]
2	82/M	DLBCL	CD4+, CD5+, CD19+, CD20+, CD23+, CD25+, kappa+			R-CHOP × 5	Complete remission after chemotherapy	Left cervical and left inguinal lymph node		[11]
3	55/F	DLBCL	CD20 (weak), BCL2, PAX5, surface kappa, MUM1, and CD4	Clonal *IgH* gene rearrangement and no *BCL2* gene rearrangement	Uniformly soft, greyish tissue	Mega Chop; R-Mega CHOP × 5	Complete remission after chemotherapy	Ileum		[6]
4	73/M	DLBCL	CD45 (dim), CD19, PAX5, CD20, CD10, BCL6, BCL2, surface lambda light chain, and CD4	Clonal *IgH* gene rearrangement and a *BCL2* gene rearrangement		Adjuvant chemotherapy		Ileum		[6]

CD: Cluster of differentiation; DLBCL: Diffuse large B-cell lymphoma; F: Female; M: Male; N/A: Not available; CHOP: Rituximab, cyclophosphamide, doxorubicin hydrochloride, vincristine sulfate, mega.

adrenal gland and superior pole of the right kidney. Retroperitoneal lymph-node fine needle aspiration showed numerous large lymphoid cells with centrally-located nucleoli and vesicular chromatin.

Aberrant expression of CD4 in DLBCL is a rare but intriguing finding that raises questions of lineage fidelity and the biology underlying such aberrant expression. Under physiological conditions, lineage commitment and differentiation are considered unidirectional and irreversible processes[12]. That is to say, a lymphocyte committed to the B-cell program is destined to become a B-cell, one that we do not normally expect to dedifferentiate to an immature form. So how then does one explain aberrant T-cell antigen expression in B-cell lymphomas? Various hypotheses, some of which challenge this paradigm, have been advanced to account for this unusual phenomenon.

Some believe that aberrant expression is the result of neoplastic transformation at stem cell level before commitment to either B or T-cell differentiation[13,14]. Others postulate expansion of a normal subpopulation expressing T cell antigens [$CD5^+$ B1 cells, CD2(+) B cells, CD7(+) B cells][8,15]. It is also possible that deregulated control of gene expression in malignant B cells leads to the activation of some otherwise silent or repressed genes of T-cell differentiation[8,13,15-18]. For example, deregulated/damaged PAX5 might downregulate B-cell antigens and lead to aberrant expression of a T-cell antigen such as CD4. This notion is corroborated by murine models in which conditional deletion of PAX5 resulted in dedifferentiation of mature peripheral B cells into early uncommitted progenitors that were able to rescue T lymphopoiesis in the thymus of T-cell-deficient mice[12].

The import of T-cell antigen expression in B-NHL lies beyond its identification as a biological curiosity or its role in helping us understand lymphopoiesis, and in turn lymphomagenesis. There are practical implications as well. Particularly, recognition of T-cell antigen expression in B-NHL is important to avoid erroneous diagnostic consideration of a T-cell malignancy, a potential pitfall that is potentiated by sometimes weak expression of B-cell defining markers. Given that T-cell markers are not routinely performed in cases of DLBCL, the actual incidence of CD4 positivity may actually be higher than cited in the literature. However, results from sizeable series looking at T-cell antigen expression in B-NHL would argue against this possibility and suggest that CD4 expression is in fact rare[3].

We must also consider whether there are any prognostic implications. The data are not entirely clear in this regard. There are isolated reports showing worse outcomes for T-Ag (+) B-NHLs. Some report increased extranodal involvement and a higher International Prognostic Index (high and high intermediate) score in such cases[3]. On the other hand, Olack *et al*[8] did not find any difference when T-Ag (+) B-NHLs were compared to their normal B-NHL counterparts. With regards to our series of DLBCLs expressing CD4, it appears that at least a subset may behave aggressively based on our data. In summary, aberrant CD4 expression in ALK-negative DLBCL is a rare, but documented, phenomenon that raises interesting biological and diagnostic considerations.

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Modified skin bridge technique for ilio-inguinal lymph node dissection: A forgotten technique revisited

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Abstract

Ilio-inguinal lymph node dissection (IILD) is a commonly performed surgical procedure for a number of malignant conditions involving mainly the male and female genitalia, and the skin; however the postoperative morbidity of IILD, due to high frequency of flap necrosis, wound infection and seroma formation, has always been a major concern for the surgeons. The aim of the study is to highlight a modified skin bridge technique of IILD using two parallel curvilinear incisions to minimize postoperative skin flap necrosis. This technique was successfully employed in 38 IILD during May 2012 to November 2013. None of the patient had flap necrosis. Two patients developed seroma while another two patients had superficial surgical site infection; they were managed conservatively. Modified skin bridge technique for IILD is an effective method to minimize flap necrosis without compromising the oncological safety.

Key words: Skin bridge technique; Wound infection; Ilio-inguinal lymphadenectomy; Flap necrosis

Core tip: Ilio-inguinal lymph node dissection (IILD) has classically been associated with high postoperative complications which include flap necrosis, wound infection, seroma formation, and lymphedema. A modified skin bridge technique of performing IILD aims to minimize the postoperative wound complications. It is an effective method to minimize flap necrosis without compromising adequate oncologically safe dissection.

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INTRODUCTION

Ilio-inguinal lymph node dissection (IILD) has classically been associated with high postoperative complications which include flap necrosis, wound infection, seroma formation, and lymphedema. Although a number of modifications of surgical techniques to perform IILD have been reported in the literature, frequency of various complications remains high. In a recent review of IILD, Hegarty *et al*[1] reported that frequency of skin flap necrosis varies from 7.5% to 61% in radical IILD; other major complications of IILD include infection (7.5% to 14.2%), seroma formation (5% to 13.8%) and lymphedema (2.5% to 5.2%). In 1972, Fraley *et al*[2] described a new technique "skin bridge technique" for IILD to reduce postoperative wound complications; however, this technique failed to gain popularity among the surgeons. We, herein, present our modified skin bridge technique which aims to lessen the frequency of postoperative skin flap necrosis.

OPERATIVE TECHNIQUE OF MODIFIED SKIN BRIDGE TECHNIQUE

The patient was positioned supine with abduction and external rotation at hip joints (Figure 1). This technique entailed two curvilinear incisions: An inguinal incision, of approximately 7 cm length, 4 cm below and parallel to the inguinal ligament; and an iliac incision, of 5-7 cm length, 4 cm above and parallel to the inguinal ligament (Figure 2A). During the inguinal part of dissection, the skin flap was raised preserving the subcutaneous fat, just superficial to Scarpa's fascia, with flap (Figure 2B); the lateral limit of dissection was the medial border of Sartorius and the medial limit was the lateral border of adductor longus, the upper limit was 2-3 cm above the inguinal ligament, and the lower limit was the apex of the Scarpa's triangle. During the iliac part of the dissection - the external oblique muscle was divided along the skin incision, and the internal oblique and transverses abdominis muscles were split along the muscle fibres. The retroperitoneal space was entered thereafter. Inguinal and iliac lymphadenectomy is completed as per the standard technique. The margins of the inguinal incisions were routinely freshened before closure. Sartorius flap was routinely used to cover the femoral vessels in all patients. One closed suction drain was placed in the inguinal region; no drain was used for iliac dissection area (Figure 3). A light pressure dressing was done. All the patients were prescribed elastic stockings within eight hours of the surgery.

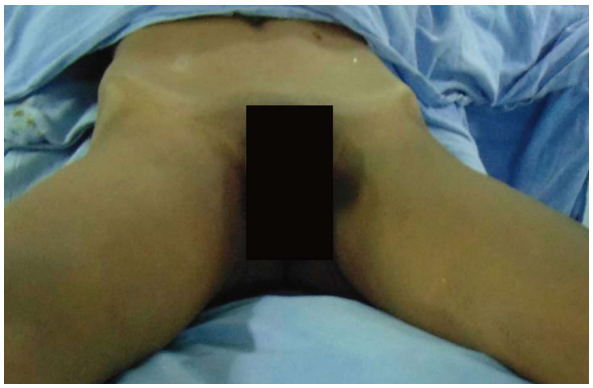

Figure 1 Position of the patient, supine with abduction and external rotation at both hip joints.

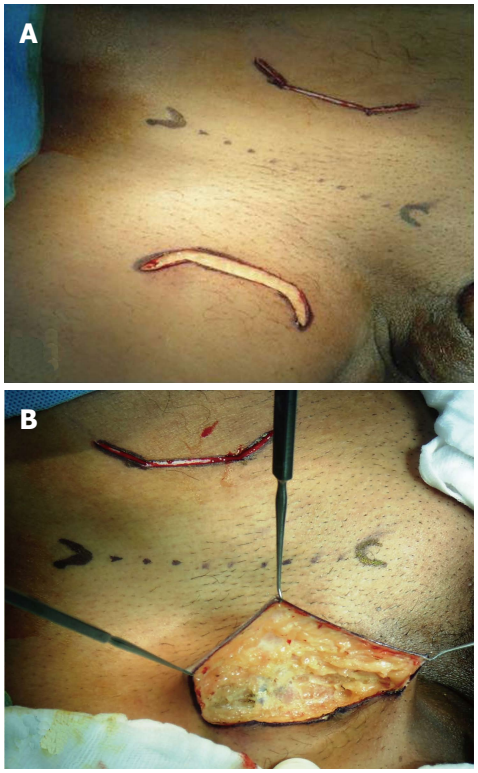

Figure 2 Two parallel curvilinear incisions technique: One inguinal incision, another iliac incision (A), and during inguinal part of dissection, the skin flap is raised preserving the subcutaneous fat, superficial to scarpa's fascia (B).

Antithrombotic prophylaxis was not routinely prescribed. The patients were discharged on 2nd postoperative days after having been explained and taught surgical site care and how to measure daily drain output. The drain was removed in an out-patient clinic after the drain output decreased to less than 40 mL/d. All patients receive perioperative antibiotics for five days or till drainage tube was not removed.

RESEARCH RESULT

This technique was successfully employed in 38 IILD

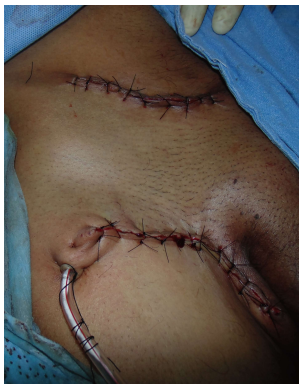

Figure 3 Immediate postoperative photograph.

during May 2012 to November 2013. There was no perioperative mortality. None of the patient had flap necrosis. Six patients developed seroma while another two had superficial surgical site infection; they were managed conservatively. For a unilateral ilio-inguinal lymphadenectomy, the median lymph nodes harvested were 10 (interquartile range 7-15) while the median operative time and blood loss was 50 min (interquartile range 45-60) and 15 mL (interquartile range 10-25) respectively.

DISCUSSION

IILD has been traditionally an integral part of management of male and female genital cancers, and skin cancers; however, it is associated with considerable post operative morbidity and high frequency of ischemic necrosis of skin flaps. These complications have reduced over a period of time with certain technical modifications as it is evident from two publications from a single institute in south India[3,4]. In a large series of 174 IILDs, performed from 1962 to 1990, Ravi[3] reported that postoperative complications of IILD were partial skin flap necrosis in 64%, wound infection in 14%, seroma formation in 9%, and limb lymphedema in 29% of dissections. Another paper form the same institute[4], published 14 years later highlighted a lower rate of flap necrosis (19.8%) in 202 IILDs performed from 1987 to 1998. Though there was a reduction in the rate of flap necrosis in IILDs, it continued to be considerably high (19.8%). The high postoperative morbidity associated with IILD is at the helm of many controversies: Whether the extent of lymphadenectomy can be curtailed in early cancer? Whether should one go for sentinel node biopsy? Whether there is any role for prophylactic lymphadenectomy at all? And what is the role of whole gamut of new minimally invasive techniques of IILD. We tried to address to problem of skin flap necrosis with our innovative modified skin bridge technique using two parallel curvilinear incisions.

The blood supply of the skin flap is derived from the three superficial branches of the femoral artery namely - superficial external pudendal, superficial epigastric and superficial circumflex iliac. These vessels are usually divided during conventional technique of groin dissection, and so the vascularity of the skin flaps rely solely upon the anastomotic branches of these vessels forming a microvascular arcade which lies in the camper's fascia and runs parallel to inguinal ligament[5]. This anatomical fact is the basis of our technique as both incisions remain parallel to the inguinal ligament, and thus spare the above mentioned vessels which run superficial to Scarpa's fascia. The curves at the ends of the curvilinear incisions define the medial and lateral boundary of the dissection; an overzealous dissection beyond the limits has been a crucial factor which can jeopardize the vascularity of skin flaps and result in its necrosis[3].

There are some minor modifications that we made in our technique compared to what was described originally by Fraley and Hutchens[2]: (1) relatively smaller skin incisions; (2) routine transposition of Sartorius muscle to cover the femoral vessels; (3) no routine femoral hernia repair; (4) no *en bloc* dissection of iliac and inguinal lymph nodes; and (5) routine freshening of both skin edges of inguinal incision.

CONCLUSION

Modified skin bridge technique for IILD is an effective method to minimize flap necrosis without compromising oncological safety.

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ORIGINAL ARTICLE

Basic Study

Adjuvant role of *Pseudomonas* flagellin for *Acinetobacter baumannii* biofilm associated protein

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Author contributions: Rasooli I contributed to the conception and design of the study, or acquisition of data, or analysis and interpretation of data; Sefidi MD carried out the experimental part of the project; Owlia P revised it critically for important intellectual content; Talei D carried out the statistical analyses; Astaneh SDA supervised the experiments; Nazarian S drafted the article; and final approval of the version to be submitted was made by Rasooli I.

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Institutional animal care and use committee statement: The animal care protocol was approved by Shahed University. 4-6 wk old (16-22 g) BALB/c mice procured from the Razi Institute, Tehran, Iran were housed in clean standard animal care facility of Shahed University. The research was carried out in compliance with the Animal Welfare Act and regulations related to experiments involving animals. The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (23 ℃, 12 h/12 h light/dark, 50% humidity, ad libitum access to food and water) for two weeks prior to experimentation. All animals were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for tissue collection.

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Abstract

AIM

To study immunogenicity of *Pseudomonas* N terminal flagellin as an adjuvant for *Acinetobacter baumannii* (*A. baumannii*) biofilm associated protein (Bap).

METHODS

The N terminal flagellin gene was amplified. The pET28a (+) and polymerase chain reaction products were

digested with Hind Ⅲ and EcoR Ⅰ. The ligation of N terminal flagellin into pET28a (+) was performed using T4 DNA ligase and was then transformed into *Escherichia coli* BL21 (DE3) as a suitable expression host. pET28a (+) vector harboring a conserved region of Bap from our previous work was used. The recombinant proteins were expressed, analyzed by SDS-PAGE method and was purified by affinity chromatography with His-Tag residues followed by confirmation with western blotting. Mice were immunized with recombinant N terminal flagellin and Bap subunits. The immunized animals were intranasally (i.n) challenged with *A. baumannii* and *Pseudomonas aeruginosa* (*P. aeruginosa*).

RESULTS

The flagellin enhanced the immunogenicity of Bap causing an increase in specific IgG titers in serum ($P < 0.001$). Internal organs, *i.e.*, liver, lung and spleen of the Bap-Flagellin immunized group challenged with *A. baumannii* showed significantly lower bacterial load compared to the control group. The bacterial loads were studied in internal organs. *A. baumannii* infected immunized animals with Bap-Flagellin exhibited internal organs with minor bacterial load while *P. aeruginosa* PAO1 infected group showed heavy bacterial load of $(4.3 \pm 0.12) \times 10^6$, $(1.1 \pm 0.01) \times 10^6$ and $(2.2 \pm 0.22) \times 10^6$ per gram of lungs, liver and spleen respectively. Bacterial loads were detected per gram of lungs, liver and spleen of the mice group immunized with Bap were $(1.2 \pm 0.06) \times 10^7$, $(11.1 \pm 0.041) \times 10^5$ and $(3.6 \pm 0.42) \times 10^6$ respectively. *In vivo* neutralization assay indicated that all experimental mice groups, except for Flagellin administered group was significantly ($P < 0.05$) protected against *A. baumannii*.

CONCLUSION

These results demonstrate that *P. aeruginosa* Flagellin as an adjuvant for Bap*A. baumannii* could be a useful model to evaluate new vaccine against *A. baumannii*.

Key words: *Acinetobacter baumannii*; *Pseudomonas aeruginosa*; Vaccine; Immunogen; Biofilm associated protein

Core tip: The increasing frequency of *Acinetobacter baumannii* (*A. baumannii*) infections and its drug resistance challenge health authorities. Flagellin is an effective immune activator stimulating various biologic functions identified by Toll like receptor 5. Conserved regions of biofilm associated protein (Bap) have already been identified and their immunoprotectivity against *A. baumannii* have been established. In order to enhance their immunogenic activities, we designed a study on adjuvant role of flagellin from *Pseudomonas aeruginosa* for Bap.

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INTRODUCTION

Acinetobacter baumannii (*A. baumannii*) an opportunistic pathogen, causes severe infections of major concerns[1,2]. It survives on harsh dreadful environment such as medical devices. This ability has been related to forming multicellular complex named biofilm on abiotic and biotic surfaces[3,4]. The *A. baumannii* survival has also been contributed with clinical strains resistance to antimicrobial stressors and desiccation[5,6]. Formation by *A. baumannii* clinical strains of biofilms on abiotic surfaces has been documented[5]. Production of factors like poly-β-1,6-*N*-acetyl glucosamine (PNAG), in different bacteria that produce PNAG could also act as a major virulence factor for emerging biofilm-dependent pathogens[7]. Biofilms are encapsulated bacterial constructions within exopolysaccharide, a polymeric matrix important in medicine[8]. Identifying a biofilm associated protein (Bap) homologue of staphylococcus[9], in an isolate of *A. baumannii* from bloodstream showed that Bap is the most important factor in *A. baumannii* biofilm formation[10]. The role of high molecular weight of superficial Bap conferring biofilm formation capacity upon bacterial attachment and infection has been reported[10]. Bap *A. baumannii* is one of the biggest and the most acidic bacterial proteins with a predicted pI-3 is composed of about 8620 amino acids(aa). Seven tandem repeats are the major functional and conserved regions components of Bap[11]. Using adjuvants and immune modulators during vaccination helped to increase immune responses[12]. Adjuvant can enhance host response against an immunogen or a vaccine without imparting antigenic effect. Various substances acting as vaccine delivery vehicles such as mineral salts, particulate and surfactants or liposomes and virosomes have been extensively used as adjuvants[13]. Pattern recognition receptors called "Toll like receptors (TLRs)" are present on various types of microbial cells[14]. TLR5 recognizes flagellin, a potent immune activator stimulating diverse biological functions. In a classic study, a recombinant fusion protein strategy was used with TLR5s like Flagellin to show TLR5 role in the development of vaccine[15]. N-terminal region amino acids of *Pseudomonas aeruginosa* (*P. aeruginosa*) PAO1 flagellin was administrated to play significant binding role to TLR5. Specific involvement of different amino acids in TLR5-flagellin interactions was particularly predicted[16], so researches show significant role of N-flagellin terminal region in immunization and can play as well as whole flagellin. The present study was designed to examine the role of *Pseudomonas* flagellin as an adjuvant to Bap

against infections caused by *A. baumannii*.

MATERIALS AND METHODS

Materials

The kits for extraction of plasmids for purification of gels were purchased from a local dealer. Synthesis of the designed primers was done by Gene Fanavaran (Tehran, Iran). Standard quality restriction enzymes, Nickel Nitrilotriacetic Acid (Ni-NTA), T4 DNA ligase, nitrocellulose membrane, anti-polyhistidine antibodies, anti-mouse HRP conjugated IgG, microtiter plates, and other standard chemicals and reagents were procurred from local market.

Bacteria

A. baumannii (ATCC 19606), *P. aeruginosa* (PAO1) and *Escherichia coli* (*E. coli*) BL21 (DE3) grown in Luria Bertani (LB) medium on a shaker at 37 ℃ /220 rpm were used.

Gene amplification and plasmid construction

The N terminal Flagellin gene (Gen Bank accession No.: AGY69124.1) with 1-180aa of the mature Flagellin protein weight of 53.79 kDa from *P. aeruginosa* was amplified by polymerase chain reaction (PCR) using its genomic DNA. N terminal F (5'-ATATGAATTCATGGCCCTTACAGT-3') and N terminal R (5'-TATAAAGCTTTTAACCGCTGATCT-3') primers. The F primer contained EcoRI site and R primers had HindⅢ site. PCR conditions started at 95 ℃/ 5 min followed by 35 cycles at 94 ℃/30 s, 58 ℃/1 min, 72 ℃ /90 s, and a 72 ℃ for 5 min as the final extension. One percent agarose gel was used to analyze the amplified fragments. Double Digestion of PET28a (+) vector and PCR products was carried out and T4 DNA ligase was used for ligation of N terminal Flagellin into pET28a (+). Transformation of the recombinant DNA plasmids, N Flagellin, pET28a into *E. coli* BL21 was then carried out.

Protein expression and purification

Vector harbouring pET28a-n Flagellin construct was incubated for 3 h at 37 ℃ in LB agar incorporated with 70 μg/mL kanamycin to an OD_{620} of 0.6. Induction was brought about with 1 mmol IPTG for 3 h at 37 ℃. The cell pellet was collected after 10 min centrifugation at 5000 rpm and re-suspended in denaturation lysis/ binding buffer (buffer B). Lysate sonication at 200 w with a 10 s cooling time between each burst was done for 6 times. The lysate was then centrifuged at 14000 rpm at 4 ℃ for 20 min to pellet the cell debris. The affinity Ni-NTA agarose column loaded with supernatant was washed with denaturing buffer (buffer C, pH = 6.3), and denaturing elution buffer D (pH = 5.9). The recombinant Bap subunit was then eluted using denaturing elution buffer E (ph = 4.5). Eight mol/L urea was present in all buffer solutions. Protein analysis was carried out by 10% SDS-PAGE. Dialysis was performed in descending order against PBS (pH = 7.4) containing 6 to 0 mol/L urea. Huleatt *et al*[15] method was employed to determine the purified protein concentration. Bovine serum albumin (BSA) was used as a standard protein. Production of the recombinant Bap and its and purification was according to Fattahian *et al*[17].

Western blotting

Western blotting was performed to confirm the expression of N-flagellin and Bap. Anti-His. 0.5 mg from each of purified recombinant Bap subunit was adsorbed on a nitrocellulose strip and dried. The transformed lysates from uninduced cell were used as control. The nitrocellulose strip was incubated for 1 h at room temperature with gentle shaking in the blocking buffer containing 5% skim milk. The strip was then subjected to three washes with phosphate buffered saline containing Tween 20 (PBST). Diluted anti-His conjugated was incubated with 1:8000 dilution of horseradish peroxidase for one hour. The strip was then subjected to three washes of 5 min each with PBST. Membrane visualization with the substrate diaminobenzidine was complete by observing brownish dots. Color development was terminated by washing with PBST.

Animal immunization

Sixty BALB/c mice were divided equally in six groups: (1) the first group was administered with four 20 μg doses of the recombinant Bap; (2) the second group was administered with four 20 μg doses of the recombinant N Flagellin; (3) a single 20 μg dose of recombinant Bap followed by administration of N-flagellin in subsequent vaccinations was administration plan for the third group; (4) the fourth group received a single dose of 20 μg of recombinant N-flagellin followed by administration of Bap in subsequent vaccinations; (5) the fifth group received combination of Bap and N-flagellin simultaneously in all the vaccinations; and (6) the control group received combination of PBS and Freund's adjuvant.

All groups except group (5), first received complete Freund's adjuvant only The subsequent vaccinations were with the incomplete Freund's adjuvant emulsified recombinant proteins. Ten days after the second dose the blood samples were collected through infra-orbital plexus.

ELISA with recombinant Bap and N-flagellin subunit

The coating solution was used to dilute the recombinant Bap and N-flagellin subunit to an optimal concentration of 20 μg/mL to coat a 96-well plate. Each well was added with 100 μL of the resulting solution followed by incubation at 4 ℃ for 12-18 h. The wells were washed once with 0.05% Tween 20 (PBST) incorporated PBS to block the unoccupied sites. The plate was then incubated for 1 h at 37 ℃ with 100 μL of PBST + 5% skimmed milk. The plates were washed 3 times with PBST. Serial dilutions of 1:100 to 1:1600 from each serum were added to the wells. The plates were incubated at 37 ℃ for 1 h followed by 3 washes as described above. One hundred microlitre of 1:1000 horseradish peroxidase

- conjugated secondary antibody diluted in PBST was added to each well and the plates were incubated at 37 ℃ for one hour followed by three washes with PBST and incubation with 100 μL per well of TMB (3,3′,5,5′-tetramethylbenzidine solution) substrate until a desired absorbance was reached. Addition of 2 mol/L H_2SO_4 stopped the reaction. Sample absorbance at 450 nm was read on an ELISA plate reader.

Cell ELISA

The clinical *A. baumannii* isolates incubated overnight in LB broth were resuspended in PBS to an OD_{620} of 0.3[17]. Fifty microlitres of the bacterial suspension was added to each well in 96-well plates and dried at 37 ℃. The plates were washed five times with after blocking with 200 μL of PBS with 5% skimmed milk for 1 h. This was followed by incubation with different dilutions of immunized mice sera. The plates were then incubated with HRP conjugated secondary antibody for one hour. PBS was used to wash the plates for five times. One hundred microlitres of TMB substrate was added per well and incubated until desired absorbance. The reactions were stopped with 2 mol/L H_2SO_4 and OD_{450} was read.

Fifty percent lethal dose (LD_{50}) determination

Cyclophosphamide (Cy) regimen (150 mg/kg of body weight) was used to bring about neutropenia of short duration but able to facilitate the onset of the infectious process. LD_{50} was determined with intranasal (i.n) administration of *A. baumannii* at 10^9 CFU concentration 4 d after treatment of five BALB/c mice with Cyclophosphamide. PBS was administered as a control instead of bacteria. General anesthesia was brought about by intraperitoneal (i.p) injection of 0.2 mL of 1.3 mg/mL xylazine and 6.7 mg/mL of ketamine in 0.9% saline. This was 100-200 mg Ketamine and 5-16 mg Xylazine per kilogram body weight.

Neutralization assay

Antisera to the Bap and Bap-Flagellin proteins were raised by injecting 20 μg of Bap and Bap-Flagellin per animal in BALB/c mice. Lethal dose (10^6 CFU) of *A. baumannii* diluted to 1:400 with PBS was maintained at 37 ℃ for 30 min. Neutralization test was carried out by peritoneal injection of lethal dose of *A. baumannii* to the mice groups of five animals per group. In order to rule out interfering role of natural antibodies in mice sera in conferring any resistance to mice against *A. baumannii*, a lethal dose of *A. baumannii* and PBS was administered to mice groups as control[18]. Mortality rate was monitored for 48 h.

Challenge of mice

Mice immunized with Bap and Bap-flagellin were divided into two groups. The groups were challenged with *A. baumannii* and *P. aeruginosa* PAO1. The mice were observed for mortality for two days. The animals were sacrificed after 48 h unless they died earlier. The microbial challenge or passive immunization were performed in mice groups treated with Cy. For bacterial challenge, i.n. administration with 20 μL of *P. aeruginosa* PAO1 or 20 μL of *A. baumannii* (10 μL/nostril) was carried out[19]. Morbidity and mortality were watched over 48 h.

Statistical analysis

The experimental data in triplicates were expressed as mean ± SD. In order to calculate *P* values and to determine the significance of differences in the experimental groups, Student's *t* test was used. $P < 0.05$ was considered as significant for the combined injection of Bap and Flagellin.

RESULTS

Bap and N terminal flagellin gene

N terminal flagellin gene of *P. aeruginosa* (PAO1) was successfully amplified. The amplified gene was revealed on agarose gel (1%). N-flagellin gene was cloned into pET28a (+). The N-flagellin and Bap were expressed in *E. coli* BL21 (DE3). The recombinant proteins were confirmed by SDS-PAGE. The presence of 42 kDa (Bap) and 7 kDa (Flagellin) proteins in the eluted fractions was revealed by SDS-PAGE analysis. Western Blotting was used to confirm the expression of recombinant proteins using anti-His-tag antibodies (Figure 1).

Recombinant proteins' immunogenic properties

Animals were observed healthy with no post immunization signs of abnormalities. Significant ($P < 0.05$) levels of IgG were noted in immunized sera compared to control mice. Increased antibody titer was noted after the third booster dose. No specific antibody was detected in sera samples from mice that received adjuvant and PBS. No significant difference was observed in the sera from combined administration of both proteins as compared to that of the single Bap injections (Figure 2A and E). Significant ($P < 0.001$) increase of antibody titer took place after the third booster, whereas animals administered with adjuvant and PBS or just Flagellin serving as control exhibited no Bap-specific antibodies in their sera.

Cell ELISA

The antibody raised against Bap subunit reacted with *A. baumannii*. OD values detected were significant compared to control (Figure 3).

Animal challenge with A. baumannii and P. aeruginosa

2.5×10^9 CFU/mL was determined as LD_{50} per mouse *via* intranasal administration. Rendering mice immunocompromised by prior treatment with Cy makes them susceptible to pneumonia. This susceptibility is accompanied by a drop in the LD_{50} after a challenge with 1000 CFU of a *P. aeruginosa* PAO1 or *A. baumannii* causing lethality. The control group died within the first 24 h of challenge. The Bap immunized groups resisted *A.*

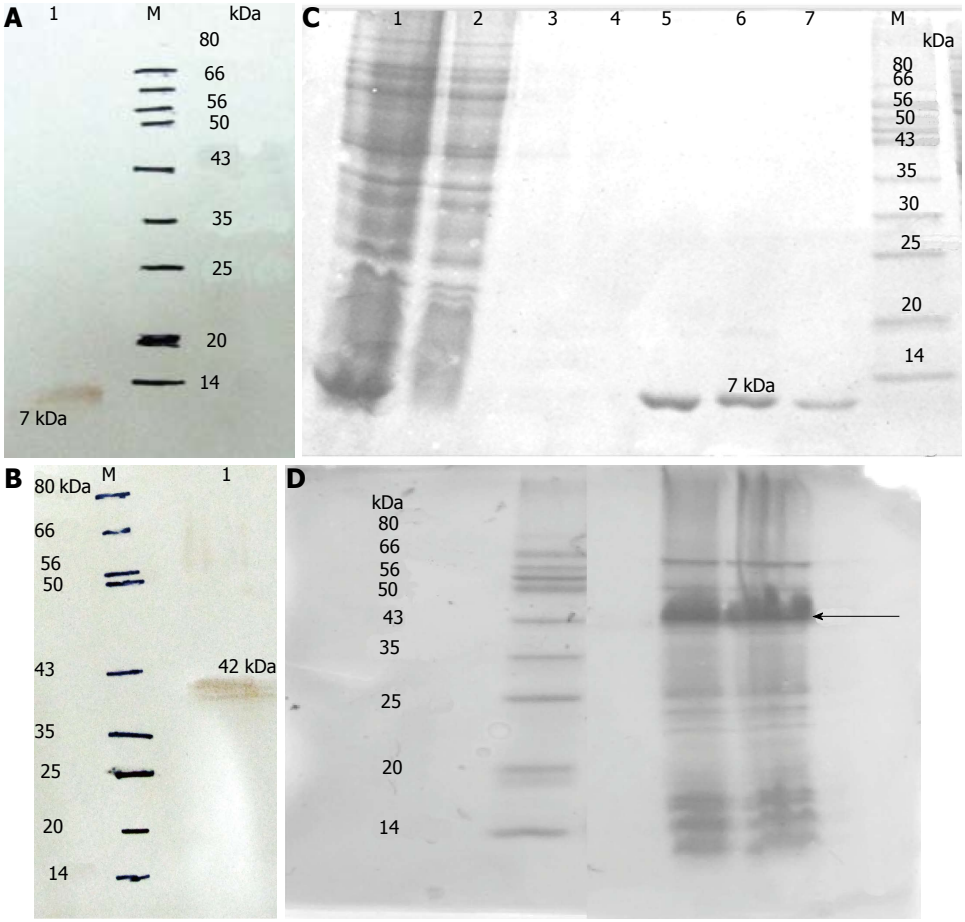

Figure 1 Western blot analysis of (A) 7 kDa recombinant N Flagellin, and (B) 42 kDa Bap subunit, (C) and (D) sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis revealing the presence of 7 kDa Flagellin and 42 kDa biofilm associated protein.

baumannii challenge with no mortality. Twenty percent of the mice population immunized with Bap died upon challenge with *P. aeruginosa* within 48 h. Eighty percent of the group immunized with Bap-Flagellin survived challenges with *A. baumannii* or *P. aeruginosa* (Figure 4).

Immunization and bacterial uptake in lungs, liver and spleen

The internal organs were studied for bacterial load. *A. baumannii* infected immunized animals with Bap-Flagellin exhibited internal organs with minor bacterial load while *P. aeruginosa* PAO1 infected group showed heavy bacterial load of (4.3 ± 0.12) × 10^6, (1.1 ± 0.01) × 10^6 and (2.2 ± 0.22) × 10^6 per gram of lungs, liver and spleen respectively. Bacterial loads in terms of CFU/g of lungs, spleen and liver of the mice group immunized with Bap were (1.2 ± 0.06) × 10^7, (11.1 ± 0.041) × 10^5 and (3.6 ± 0.42) × 10^6 respectively. Unimmunized mice exhibited bacterial load of (3.20 ± 0.11) × 10^7, (1.90 ± 0.14) × 10^6 and (2.6 ± 0.11) × 10^6 CFU per gram of lungs, liver and spleen respectively in challenge with *A. baumannii* and (2.17 ± 0.2) × 10^7, (2.1 ± 0.1) × 10^6 and (5.4 ± 0.11) × 10^6 per gram of lungs, liver and spleen respectively in challenge with *P. aeruginosa* PAO1 (Figures 5 and 6).

Neutralization assay

Protectivity of immunized mice sera against bacterial challenge was determined by neutralization assay. There was an increased antibody titer against Bap and Bap-flagellin in the vaccinated group. As shown in Figures 4 and 5, all the experimental groups, except for Flagellin administered group were significantly ($P < 0.05$) protected against *A. baumannii*.

DISCUSSION

Despite development of new generation antibiotics, the wide expansion of multi drug resistant *A. baumannii* is still considered as a potent threat[20]. Because of it is remarkable resistance to an extensive range of antibiotics[21], *A. baumannii* is regarded as a problematic pathogen. Its colonization ability, survival on nutrient-limited surfaces and resistance to antimicrobial situation made it as a difficult-to-treat nosocomial pathogen[5]. Combination of biofilm forming and MDR of *A. baumanni* contribute in importance of this pathogen in Hospital - acquired infections[5]. Moreover recent researches confirm beside biofilm forming, multi drug resistance of embedded bacteria in matrix of biofilm is showed[22,23]. Using different subunits of Bap as a recombinant subunit vaccines[24], clarify the expansion of infections caused by *A. baumannii*.

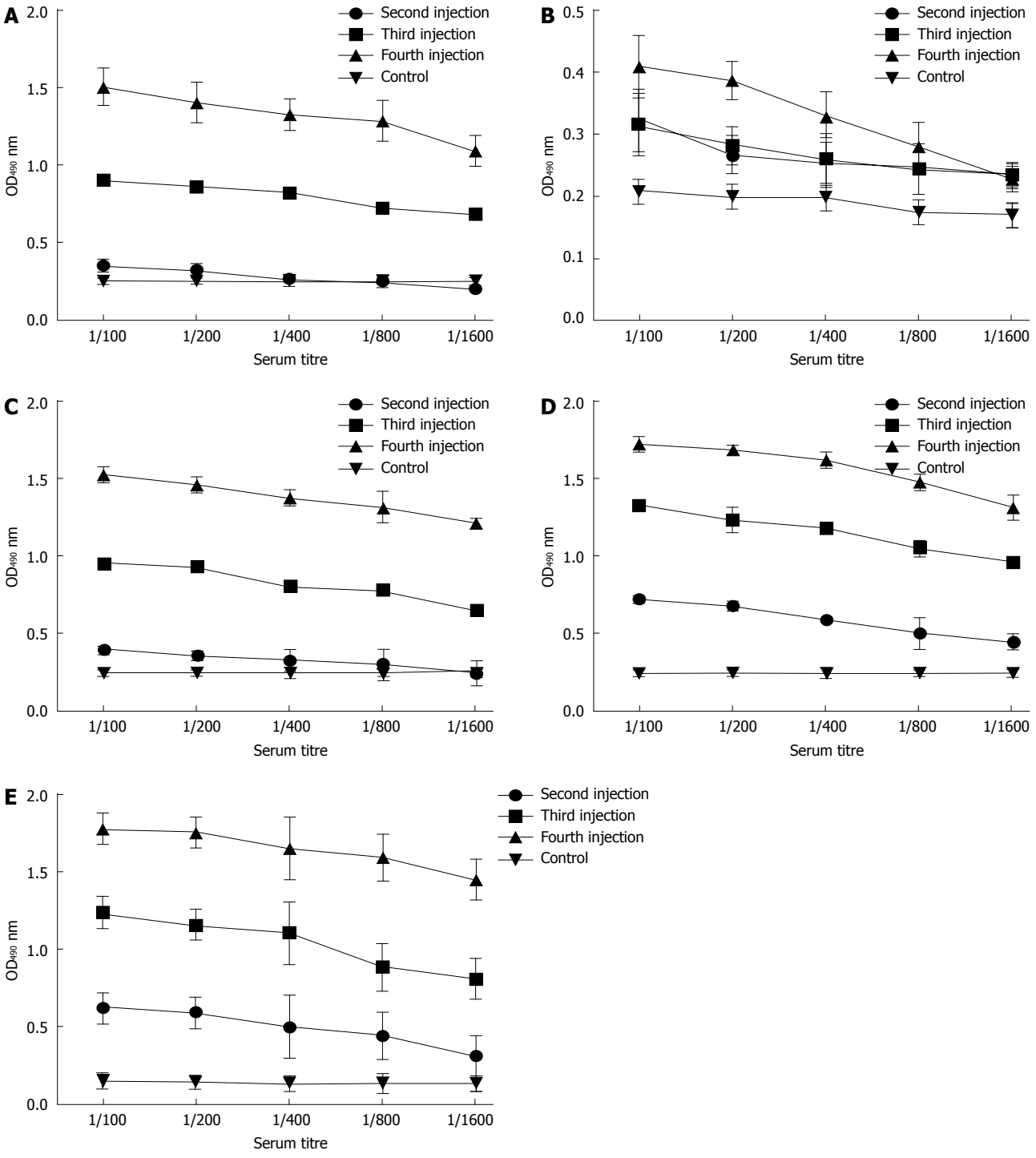

Figure 2 Indirect enzyme linked immunosorbent assay of sera of mice injected. A: Indirect ELISA of sera of mice injected with Bap: Rise of antibody titers is significant ($P < 0.001$); B: Indirect ELISA of sera of mice injected with N-flagellin subunit only: No significant rise of antibody titers was noted; C: Indirect ELISA of sera of mice injected with a single dose of Bap first followed by administration of N-flagellin in subsequent vaccinatio: Significant ($P < 0.001$) rise of antibody titers was noted; D: Indirect ELISA of sera of mice injected with N-flagellin followed by administration of Bap in the subsequent vaccinations: Significant ($P < 0.001$) rise of antibody titers was noted; E: Indirect ELISA of mice sera administered with Bap + flagellin subunit: Antibody titers increased significantly ($P < 0.001$) from the second booster onwards. ELISA: Enzyme linked immunosorbent assay.

In silico studies described intercellular adhesion of Bap*A. baumannii* in maturation of biofilm[10]. Twenty or more antigenic determinants and 55 discontinuous B-Cell epitopes were predicted for Bap subunits[11].

Adjuvant as a chemical catalyst without any considering, specific antigenic effect mount the response to a vaccine[25]. The results (Figure 2A and D) show elevated antibody level triggered against Bap indicating that N flagellin contributed to enhancement of antigen efficacy by playing a role as an adjuvant. Physical conjugation of a vaccine antigen to a TLR ligand brings about discrimination of macromolecule from self-apoptotic bodies

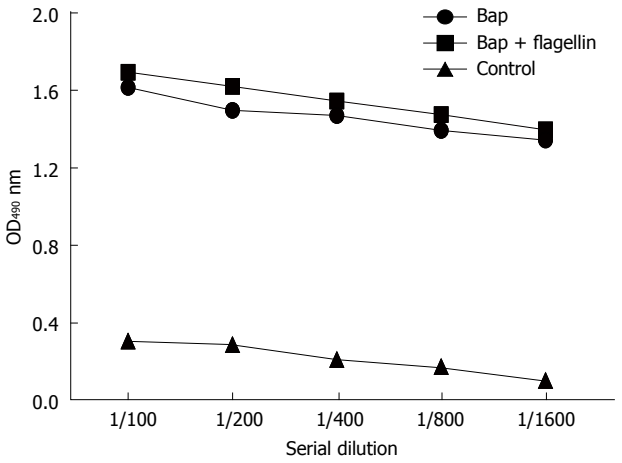

Figure 3 Whole cell enzyme linked immunosorbent assay of mice sera immunized with Bap and Bap + flagellin against *Acinetobacter baumannii*. Significant ($P < 0.05$) OD values were observed in experimental strain as compared to control group. Bap: Biofilm associated protein.

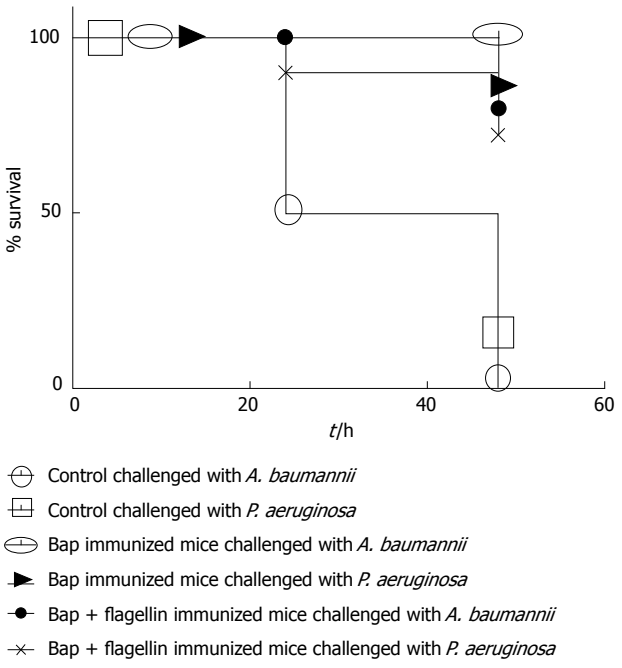

Figure 4 Survival of mice immunized with biofilm associated protein (group A) and biofilm associated protein-flagellin (group E) challenged with *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. *A. baumannii*: *Acinetobacter baumannii*; Bap: Biofilm associated protein; *P. aeruginosa*: *Pseudomonas aeruginosa*.

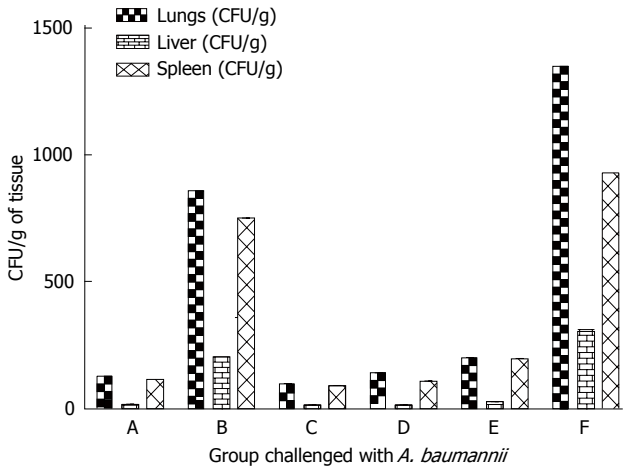

Figure 5 Neutralization. A: The group received four doses of 20 μg of the recombinant Bap; B: The group received four doses of 20 μg of the recombinant N-flagellin; C: The group received a single dose of 20 μg recombinant Bap followed by administration of N-flagellin in subsequent vaccinations; D: The group received a single dose of 20 μg of recombinant N-flagellin followed by administration of Bap in subsequent vaccinations; E: The group received combination of Bap and N-flagellin simultaneously in all the vaccinations; F: The control group received combination of PBS and Freund's adjuvant. *A. baumannii*: *Acinetobacter baumannii*; Bap: Biofilm associated protein.

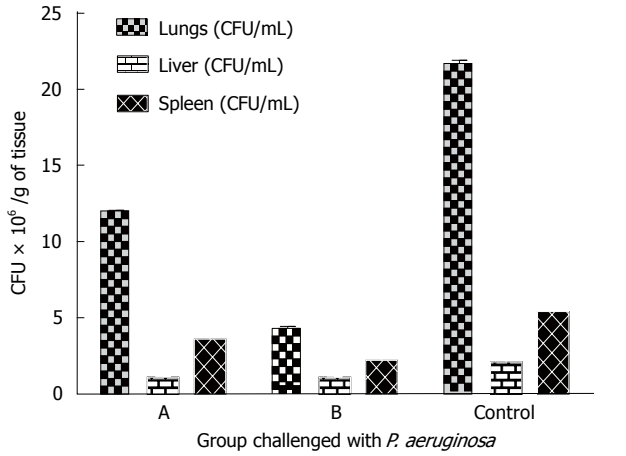

Figure 6 Neutralization test. A: The group received four doses of 20 μg of the recombinant Bap; B: The group received combination of Bap and N-flagellin simultaneously in all the vaccinations; control: The control group received combination of PBS and Freund's adjuvant. *P. aeruginosa*: *Pseudomonas aeruginosa*; Bap: Biofilm associated protein.

by the antigen presenting cells which ultimately leads to enhancement of immunogenicity and subsequently antigen presentation by MHC molecules[26]. Vance *et al*[27] investigated dissemination of *P. aeruginosa* in neutropenic mice *via* pneumonic challenge model. Bacteremia, dissemination, and eventual death of *P. aeruginosa* PAO1 challenged mice was noted upon Cy administration to the infected mice. In this work the efficacy of *A. baumannii* immunogen was studied in immunocompromised mice. Active vaccination with Bap increased survival rate and LD_{50} of Cy-treated mice to *i.n.* challenge with *A. baumannii*. Many studies described interaction of flagellin and TLR5 as a signaling of flagellin result in releasing of inflammatory mediator[28]. In silico studies describe the interaction between D1 domain of flagellin as an important region of it and TLR5[28,29]. Our study on the mice group administered only with Bap + flagellin subunit (Figure 2E) without using Freund's adjuvant showed higher antibody titer than other groups immunized with the recombinant proteins using Freund's adjuvant (Figure 2E). These results further support the adjuvant role of N-flagellin. The role of *Salmonella* Flagellin as an adjuvant has been studied. Particular interest was paid in flagellum structural subunit as an adjuvant imparting elevated immunogenicity to

soluble proteins or peptides, to activate antibody as well as cell immune responses[30]. Vaccination with chimeric flagellin provided mice with significant protection against *H. pylori*[31]. Use of EtIMP1-flagellin fusion protein has been suggested as an effective immunogen against *Eimeria* infection[32]. An experimental report on the immunogenicity of a 19 kDa merozoite surface protein-1 [MSP1(19)] from *Plasmodium vivax* C-terminal fragment against malaria and an innate immunity against the *Salmonella enterica* serovar *Typhimurium* flagellin (FliC) is available[33]. There is a report on the enhancement of FimH protective immunity against UPEC infection where the ability of FliC a Toll-like receptor 5 flagellin, a UPEC strain agonist, has been compared with Montanide ISA 206, a conventional adjuvant[34]. A TLR5 flagellin binding site at 88-97 residues located within the constant domain D1[35] coincides with that found experimentally by other researchers[28]. The importance of flagellin side chains was also described in interact with surface side chains of TLR5[35]. In this study, a 10 amino acid stretch was predicted in the N-terminal of *P. aeruginosa* PAO1 flagellin to have importance for binding to TLR5 and acting as an adjuvant[36]. Despite significant ($P < 0.05$) increase in IgG titers of the mice groups immunized with Flagellin and Bap in groups C, D and E, better protection was exhibited by Bap immunized group against lethal dose of *A. baumannii* (Figure 4). Such a phenomenon could be attributed to the vaccination dose of group E which was half of the Bap quantity used in other groups. This is further supported by the absence of initial administration of Freund's adjuvant. The adjuvant role of Flagellin subunit is well documented in groups C and D where either the initial dose of Flagellin followed by the subsequent doses of Bap or initial dose of subsequently followed by administration of Flagellin subunit significantly elevated the anti Bap titer. Vaccination of mice prior to immunocompromisation and subsequent infection of mice decreases the susceptibility on the basis of lower bacterial load and better survival rate. Significant ($P < 0.001$) rise in antibody titers of groups C (received 20 μg of recombinant Bap in the first vaccination followed by administration of N-flagellin in subsequent vaccinations) and D (received 20 μg of recombinant N Flagellin in the first vaccination followed by administration of Bap in subsequent vaccinations) and the 80% survival of group D challenged with either *A. baumannii* or *P. aeruginosa*, indicate the efficiency of Flagellin as an adjuvant and protective immunity against *A. baumannii*. The survival against *P. aeruginosa* could be due to the immunogenicity of the recombinant Flagellin.

Although there is no significant difference among groups A, C, D and E, higher load of bacteria was found in the liver and spleen of group E as compared to other immunized groups (Figures 5 and 6) which could explain the 20% mortality in this group (Figure 4).

In conclusion, because of the complications and difficulties in treating *A. baumannii* infections, and with respect to the significant role of Bap on one hand and the efficacy of bacterial proteins such as Flagellin as an adjuvant on the other, this model could be useful to evaluate new vaccine regimens against *A. baumannii* infections.

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COMMENTS

Background

Acinetobacter baumannii (*A. baumannii*) is an opportunistic pathogen of major concerns. Biofilm associated protein is the most important factor in *A. baumannii* biofilm formation. Seven tandem repeats are the major functional and conserved regions components of Bap. Pattern recognition receptors, *i.e.*, "Toll like receptors (TLRs)" are found on various microbial cells. A recombinant fusion protein strategy was used with TLR5s like Flagellin to show its role in the development of vaccine. N-terminal region amino acids of *Pseudomonas aeruginosa* (*P. aeruginosa*) PAO1 flagellin was administrated to play significant binding role to TLR5. Researches show significant role of N-flagellin terminal region in immunization and can play as well as whole flagellin. The present study was designed to examine the role of *Pseudomonas* flagellin as an adjuvant to biofilm associated protein (Bap) against infections caused by *A. baumannii*.

Research frontiers

Flagellin of *P. aeruginosa* is an important vaccine candidate. N-terminal domains are highly conserved in both type A and type B flagellins. The efficacy of gold nanoparticles (AuNPs) conjugated to N-terminal domains of *P. aeruginosa* flagellin [flagellin$_{(1-161)}$], as an immunogen in mice, has been assessed by Farida. Flagellin$_{(1-161)}$, AuNP-flagellin$_{(1-161)}$, and flagellin$_{(1-161)}$ emulsified in Freund's adjuvant were administered subcutaneously to BALB/c mice. Mice given AuNP-flagellin$_{(1-161)}$ elicited high titers of anti-flagellin$_{(1-161)}$ antibodies compared with non-immune group and/or mice which received flagellin$_{(1-161)}$ without adjuvant. Recently, Delphine demonstrated that the adaptive responses stimulated by intranasal administration of flagellin and antigen were linked to TLR5 signaling in the lung epithelium. They sought to identify the antigen presenting cells involved in this adjuvant activity. They first found that the lung dendritic cells captured antigen very efficiently in a process independent of TLR5. However, TLR5-mediated signaling specifically enhanced the maturation of lung dendritic cells. Afterward, the number of antigen-bound and activated conventional dendritic cells (both $CD11b^+$ and $CD103^+$) increased in the mediastinal lymph nodes in contrast to monocyte-derived dendritic cells. Their data suggested that flagellin-activated lung conventional dendritic cells migrate to the draining lymph nodes. The results demonstrated that indirect TLR5-dependent stimulation of airway conventional dendritic cells is essential to flagellin's mucosal adjuvant activity.

Innovations and breakthroughs

In this study the authors have used a conserved region of Bap instead of the entire protein. Bap is a very large protein and its expression is near to impossible in recombinant form. There are many such proteins involved in pathogenesis of various micro-organisms. This study can be a clue to the researchers to use immunogenic conserved regions of proteins involved in pathogenesis and enhance their immunogenicity by natural adjuvants such as flagellin.

Applications

The findings could be applied in development of novel vaccines against disease causing micro-organisms.

Terminology

TLR: "Toll like receptors" are pattern recognition receptors present on various types of microbial cells; Bap: Biofilm associated protein. Bap *A. baumannii* is one of the biggest and the most acidic bacterial proteins with a predicted pI -3 is composed of about 8620 amino acids (aa). Seven tandem repeats are the major functional and conserved regions components of Bap.

Peer-review

The authors present the extent which *Pseudomonas* flagellin can play a role as

an adjuvant for Bap *A. baumannii* and the model could be useful to evaluate new vaccine regimens against *A. baumannii*. This manuscript is interesting. It's just a suggestion, the N-flagellin and recombinant Bap could be fused to express.

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